Epidermal Growth Factor (EGF) and Transforming growth factor (TGFβ1) promote EMT in primary prostate cancer cells via Ras signaling. Inhibitory effects of EGCG on EMT induced matrix metalloproteinase (MMP) activity in prostate cancer cells.

A Thesis

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of

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Dedications

To my parents, Remya Varma and Kulasekhara Varma, to whom I owe everything I am today.

Without your words of encouragement and confidence in my abilities I could not have reached this milestone in my life.

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Abstract

Epidermal Growth Factor (EGF) and Transforming growth factor (TGFβ1) promotes EMT in primary prostate cancer cells via Ras signaling. Inhibitory effects of EGCG on EMT induced matrix metalloproteinase (MMP) activity in prostate cancer cells.

Devika Varma

Mark E. Stearns, Ph.D.

Epithelial to mesenchymal transition (EMT) may be a critical step in prostate cancer progression and metastasis. However, we do not understand the ligands and conditions controlling EMT. We have examined the factors controlling EMT in primary prostate cell lines isolated from human prostate cancer (i.e. IBC-10a and PCa-20a cells). We have shown that a combination of EGF and TGFβ1 (E+T) can promote expression of Vimentin and matrix metalloproteinases (MMP2 and MMP9) during induction of EMT in the primary cell lines. We have found that an intact Ras signaling was essential for E+T induced EMT. We have stably transfected IBC-10a cells with pBABE.ras constructs containing three distinct Ras mutations (i.e. C40, G37 and S35). In cells transfected with C40 and S35, TGFβ1 alone induced MMP2 and MMP9 secretion, whereas, E+T was essential to induce MMP2 and MMP9 secretion in G37 transfected cells (i.e. which activates RalGDS and blocks Akt-1 and MEK signaling). Taken together, the data have shown, for the first time, that E+T activation of Akt-1 and MEK signaling pathways play a key role in EMT. One added goal of the study was to identify potential therapeutic agents which can block EMT. We have found that the green tea extract, Epigallocatechin-3-gallate (EGCG), blocks E+T induced MMP secretion in a dosage

dependent manner in both the primary cell lines and malignant PC3ML2 tumor cells. In sum, our work has demonstrated that specific ligands regulate EMT and that a herbal tea extract has potential therapeutic benefit in blocking ligand dependent EMT.

Chapter 1. Introduction

Prostate Anatomy and Physiology

The prostate is a tubuloalveolar gland of the male reproductive system that lies between the bladder and the rectum in males (Figure 1). The main function of this gland is to produce the prostatic fluid, an important component of the semen, which contains several proteins vital for the survival and function of sperms. (Hayward et al, 2000) It is composed of pseudostratified columnar epithelium with basal nuclei lining the ducts. The epithelial basement membrane is further bordered by basal epithelial cells. This epithelium is surrounded by a fibromuscular stroma. (Hayward and Cunha, 2000)

The main function of the prostate is to assist the seminal vesicles in the production of the ejaculate which is primarily composed of proteins that have a variety of functions like coating and uncoating of the spermatozoa and others like prostate specific antigen (PSA). The PSA is produced by the columnar epithelial cells of the prostate and secreted into the prostate ducts. (Hayward and Cunha, 2000)

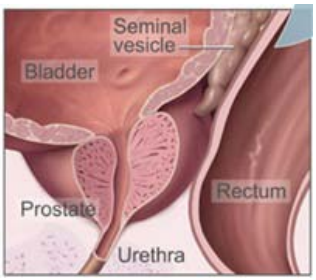


Figure 1: The location of the prostate gland. The prostate gland lies between the bladder and the rectum in males.[3]

Prostate Cancer Statistics

With an estimated mortality of close to 27,000 in 2009, prostate cancer is the leading form of cancer in the United States excluding skin cancer. This cancer has been found to affect older men who are 55 years or older. According to the National Cancer Institute, the median age at death was 80 in the United States between 2002 and 2006. [5]

Prostate Cancer Stages

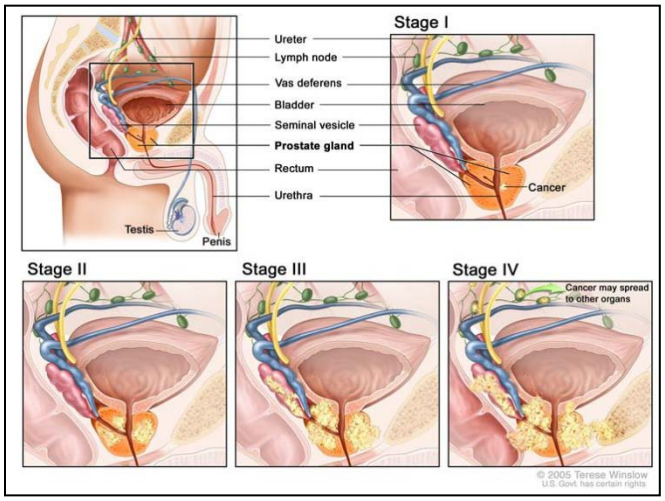


Figure 2: Stages in prostate cancer. The above figure shows the four stages in prostate cancer. (Adapted from National Cancer Institute, 2005)

Cancer stages are often represented by the Gleason's coefficient which is a score from 2-10, describing how metastatic the cancer cells are compared to normal cells. The higher numbers are associated with a higher degree of metastasis as represented by the figure above. Metastasis in prostate cancer usually involves the spread of the cancer into surrounding organs like the bladder, rectum, bone, liver or lungs. [1]

Prostate Cancer Treatment

Several methods are presently being used to identify and treat prostate cancer. The main concern lies in the early detection of the disease to prevent metastasis. The PSA test is the most common diagnostic test, though it is currently found to be inefficient in detecting budding tumors in the prostate. The detection of the PCA3 gene in urine is a newer method used for diagnosis and has been found to be more reliable. Other ways rely on imaging techniques like Color Doppler Ultrasound, enhanced MRI and CT. [1, 2]

Treatments currently employed for prostate cancer primarily include surgery, radiation therapy, high intensity focused ultrasound, hormone therapy, chemotherapy, vaccines and antibodies. Radiation therapies that entail conformal radiation therapy (CRT), intensity modulated radiation therapy (IMRT), and proton beam radiation have been found to be more localized preventing damage to the surrounding normal tissue. [2]

Epithelial to Mesenchymal Transition

The epithelial to mesenchymal transition (EMT) is a critical event in promoting the invasive capacity of malignant cells. There are several factors that have been found to transform tumor cells from its epithelial phenotype to a mesenchymal phenotype. These include ligands and signaling pathways like transforming growth factor β (TGFβ) and RTK/Ras signaling, Wnt, Notch, Hedgehog signaling pathways. Repression of genes governing an epithelial phenotype like E- cadherin by transcription factors like Snail or Slug is another means of transforming epithelial cells to a mesenchymal phenotype. (Huber et al, 2005) It is usually a combination of the above stated factors that helps induce EMT in reality. Unlike epithelial cells, which form tightly packed layers around

organs, mesenchymal cells do not adhere to each other tightly and form the inner layers of organs. Epithelial cells tend to migrate when grown in vitro and have a fibroblast and spindle shaped morphology. (Lee et al, 2006) The ability of malignant epithelial cells to acquire invasive properties requires the loss of cell junctions and the gain of mesenchymal features such as the spindle- shaped morphology. The loss of E-cadherin and ß catenin is required for this process to occur. (Lee et al, 2006) Characteristically, the EMT process is marked by the loss of E- cadherin and the increased levels of mesenchymal markers like Vimentin, Fibronectin, Snail, Slug, and Twist. Snail and Slug are the first transcription factors that were found to regulate the EMT and cell motility. (Barralo et al, 2005; Bolos et al, 2003; Cano et al, 2000; Comijn et al, 2001) Although EMT is a common event in vivo, the acquisition of this state in cells in vitro has been reported to be a rare event in a study by Moses and colleagues which showed that only a few murine cell lines were able to complete the epithelial to mesenchymal transition out of the 18 cell lines they tested using TGFβ (Brown et al, 2003). The transient reversible nature of EMT makes it difficult to observe this phenomenon in human cell lines, especially in primary cancer cell lines, where only a selective population of cells undergoes permanent EMT (Yang et al, 2006). A study in androgen refractory prostate cancer cells showed that soluble factors like EGF and TGFβ can increase bone turnover in these cells marked by the high expression of (Receptor Activator of NF-KB Ligand) RANKL, a marker indicated in prostate bone metastasis (Zhau et al, 2008).

Two zinc finger transcription factors called ZEB1 and ZEB2 are also implicated in regulating EMT by repression of E- cadherin. They function by binding to the promoter regions of E- cadherin and repressing its expression. (Comijn et al, 2001) EMT

is characterized by signals released from the tumor stroma like TGF, HGF, EGF, and PDGF leading to the activation of several transcriptional factors like Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2. The above transcriptional activation induces the activation of intracellular factors like ERK, MAPK, PI3K, Akt, Smads, RhoB, lymphoid enhancer binding factor (LEF), Ras, and c-Fos along with proteins such as β4 integrins, α5-β1 integrin, and αV-β6 integrin, which are important components of the cell surface. (Kalluri and Weinberg, 2009)

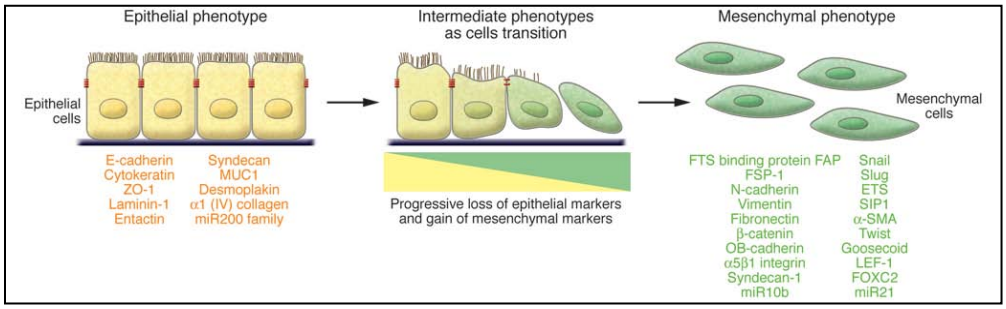


Figure 3: Steps involved in EMT. The above figure illustrates the different steps involved in the EMT process. The induction of a mesenchymal phenotype involves the expression of several genes like Slug, Snail, Vimentin, β catenin and several others. (Figure adapted from Kalluri and Weinberg, 2009).

Matrix metalloproteinases

Effective metastasis requires the degradation of the basement membrane of the primary tumor. Matrix metalloproteinases (MMPs) are endoproteins that have the ability to cleave several ECM components and are involved in matrix degradation processes during development as well as tumor invasion. Approximately 22 different MMPs have been reported, and depending on the substrate specificity have been reported to degrade

ECM components like collagen, fibronectin and laminins. These enzymes have two functional domains; a catalytic domain that consists of a Zn^{2+} binding site and COOH terminal domain that determines substrate specificity. The terminal domain, also called the hinge region is suggested to contain a Ca^{2+} binding site. A glutamine and aspartame rich region is conserved in all MMPs between the Zn^{2+} binding site and the hinge region. These enzymes remain inactive when a "pro" domain is attached to them and the catalytic activity is maintained by the "catalytic" domain. MMP2 and MMP9 have been known to degrade Type IV collagen. MMP2 resides in the ECM bound to gelatin, laminin and Type I and IV collagen. Both MMP2/9 are of particular interest in case of tumor metastasis as they are found bordering the epithelial and mesenchymal junction. It has also been shown that MMP2 is required for the activation of proMMP9. (Toth et al, 2003; Birkedal-Hansen, 1993)

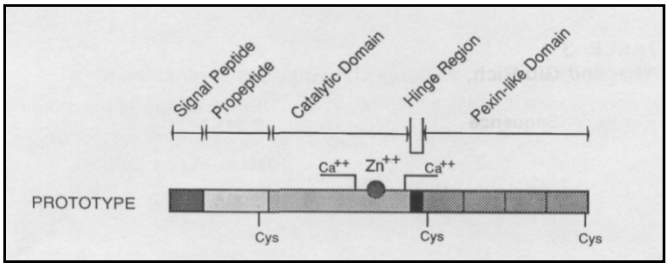


Figure 4: Prototype structure of a generic MMP. The above figure shows the main components that make up MMPs. The most important parts of this enzyme are its catalytic domain and the hinge like region. (Figure adapted from Birkedal- Hansen, 1993)

The proMMPs need to be cleaved by soluble factors or other proteases to be able to release the active forms. MMP3/ Stromelysin has been found to be the activator of proMMP9 and it cleaves the zymogen at the Glu-Met bond creating a transitional 86kDa protein. Further cleavage by MMP3 at the Arg-Phe bond results in the active MMP9 of

82kDa. (Ogata et al, 1991) The plasmin system also plays a role in creating active MMP forms by cleaving the pro forms at the right sites. Similarly, proMMP2 gets activated by the co-ordination between TIMP-2 and MMP14 (MT-MMP1) (Hernandez-Barrantes et al, 2000).

Growth factors and signaling

TGFβ one of the earliest growth factors recognized to play a prominent role in cancer progression and EMT. TGFβ1 has been found to play a dual role in cancer progression and metastasis. In the initial progression of cancer TGFβ1 has been found to act like a tumor suppressor but in the latter stages, especially during metastasis it has been found to accentuate the process. (Bierie and Moses, 2006) In non cancerous cells, TGFβ1 is a cell proliferation inhibitor and performs this function by inducing the expression of cyclin kinase inhibitors like $p15^{IKN4a}$, $p21^{K1PI25}$ and $p27^{K1PI29}$ through the Smad pathway. However, this ligand has found to stimulate proliferation in many cancer cells like those obtained from the colon, pancreas and the prostate. Binding of TGFβ1 with its type two receptor leads to the receptor interaction with occludin and is followed by the phosphorylation of Par6 protein. This step leads to the recruitment of Smurf1 which results in the degradation of Rho- a step is primarily responsible for the loss of polarity in epithelial cells. TGFβ1 also alters the gene expression of desmosomal proteins responsible for cell- cell adhesion by activating Slug. (Tsuji et al, 2009) TGFβ1has been found to regulate invasive properties of cancer cells and reduce their adhesive properties by decreasing levels of E- cadherin. It does so by inducing E-cadherin repressors like Twist1/2, ZEB1/2, Snail1/2, FOXC2 and Goosecoid that bind to the CCH1 promoter region of E- cadherin gene. (Tsuji et al, 2009) In case of normal cells, this ligand

increases the formation of the extracellular matrix, but in invasive tumors it has been found to increase protease levels.

The epidermal growth factor (EGF) primarily functions as activator for the receptor EGFR tyrosine kinase. It primarily assists the proliferation of epidermal and epithelial tissues and influences some other cell types in vivo. (Carpenter and Cohen, 1979) It plays a major role in the downstream signaling of the PI3K/ AKT pathway and the p42/44 MAPK pathway. Some studies in the past have suggested the role of EGF in increasing the invasive capacity of non neoplastic and neoplastic cells. (Lund et al, 1990) The tumor stroma often has higher levels of EGF compared to healthy tissue and is associated with inflammatory mechanisms at least in pancreatic cancer. (Farrow et al, 2004) Significant number of studies has associated an increase of MMPs in tumors with EGF and its pathways. In particular, the PI3K pathway is prominently associated with the invasive and migratory effects of EGF via NF-KB and thus indirectly affects MMP production. These results suggested in breast cell lines line up with results of other tumors like that of ovarian and pancreatic origins. (Moulik et al, 2008; Ellerbroek et al, 2001) Interestingly, studies with glioblastoma cells that compared the effects of EGF on migration and invasiveness in 2D versus 3D showed that cells grown in 3D displayed a high directional persistence (dependence on matrix dependence and protease activity) when compared to a 2D culture that showed a decrease in directional persistence. This suggests that cell intrinsic factors resulting in migration and invasion are overridden by EGF induced cell extrinsic factors that reduce matrix steric hindrance. (Kim et al, 2008) A new role of EGFR, independent of its tyrosine kinase activity was recently discovered. It has been found that EGFR directly plays a role in promoting and stabilizing the

Na/Glucose co- transporter and thus facilitating the uptake of glucose in cancer cells. (Engelman et al, 2008) Although some past studies have shown the sole effect of EGF in the promotion of migration and invasion in cancers, these effects are found to magnify drastically in the presence of TGFβ1. (Wilkins-Port et al, 2009)

Ras and its pathways

The Ras protein is found in the inner plasma membrane and its activity is regulated by alternating between the GDP bound inactive form and the GTP bound active forms. Active Ras has a number of effector molecules like PI3K, Ral, Raf and Tiam1. There is usually an over expression of tyrosine kinases in cancers, particularly of the EGF family which is found to cause a constant activation of Ras even when Ras mutations are absent.

One of most studied Ras effectors are the Raf serine/ threonine kinases. Activated Raf further activates MEK1/2 which are kinases for ERK1/2 MAPKs. The activated MAPK regulates gene expression when translocated into the nucleus. When tested in nude mice, Ras transformed cells with a Raf mutation exhibited lung metastasis which is linked to the activation of MEK by Mos, it's ectopic activator.(Posada et al, 1993) The phospoinositide 3-kinase (PI3K) is another important and well studied effector of Ras. Besides its well known role in increasing cell motility and alteration of cell- ECM interaction, it has also shown to regulate tumor cell metastasis by enabling tumor cells to escape 'matrix deprivation- induced apoptosis or anoikis. (Frisch et al, 1997) The activation of Rac GEFs (Guanine Exchange Factors) by PI3K leads to the increase in cell motility as Rac is responsible for actin reorganization and 'membrane ruffling'. (Etienne-Manneville and Hall, 2002) A recent addition to the list of Ras effectors is Tiam1, a

protein initially identified as T-lymphoma invasion and metastasis protein (Tiam1). This protein has been suggested to work in conjunction with Rac as its GEF. Several Tiam 1 knockout studies in mice resulted in drastic reduction of Rac expression suggesting the correlation between the two proteins. (Malliri et al, 2002)

Interestingly, the Met proto-oncogene plays a major role in tumor metastasis and is particularly found up regulated in tumor metastases. H-Ras transformed fibroblasts and epithelial cells express much higher levels of the Met receptor along with higher migration capabilities. (Webb et al, 1998)

Ras is the major regulator of most of the above mentioned tumor promoting actions, although the Ras pathways involved in each of these steps varies from one cell type to another. These pathways work in conjunction in areas like regulation of the actin filaments, ECM degradation, reduced cell adhesion and angiogenesis. (Campbell et al, 2004) Overall, Ras plays a very important role in tumor progression and is evidently involved in many routes leading to tumor metastasis. Ras effector pathways like ERK and Akt are well established to play a role in prostate cancer progression (Gioeli et al, 1999). One of the most recent findings about Ras signaling in prostate cancer suggests a co-operation between Ras and NF-KB to promote tumor growth and metastasis. Data suggests that the epigenetic suppression of a RasGAP gene called DAB2IP is required for NF-KB activation in prostate cancer cells leading to prostate metastasis. (Min et al, 2010)

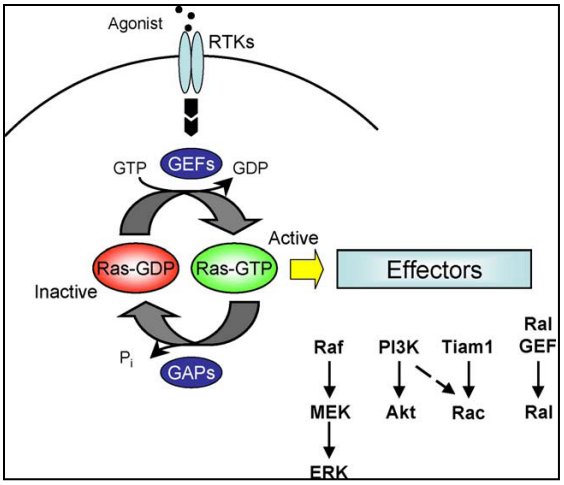


Figure 5: Important Ras pathways involved in cancer. The above image summarizes the activation of Ras with GTP binding and also shows the different effectors of Ras that further decide the fate of the cell's with respect to its invasive capabilities. Raf, PI3K, Tiam1 and RalGEF are the main effectors of Ras. (Figure adapted from Campbell et al, 2004)

Epigallocatechin-3-gallate

Innumerable cancer treatment methods have been proposed and currently several of them are being used in clinics. Antioxidants have been a major target for cancer treatments because of their ability to negate the carcinogenic effects of reactive oxygen species. Epigallocatechin-3-gallate (EGCG), an important flavanol in green tea, is an antioxidant and therefore has recently been proposed to be used in combination with other cancer drugs. (Fujiki et al, 2002) The chemopreventive activity of this molecule depicted by its ability to inhibit invasion and angiogenesis makes it useful for further investigation in the research for cancer treatment. The catechins and polyphenols found in green tea are the main components that provide this anti-carcinogenic effect. (Paschka et al, 1998) Particularly, EGCG's effect on growth inhibition of tumor cells along with its effects on invasion and migration of tumor cells have been studied in some areas of

cancer. For example, studies on prostate cancer have shown clear indication of apoptosis and growth inhibition in these cells where nuclear fragmentation and chromatin condensation was typical in the EGCG treated cells. (Paschka et al, 1998, Hastak el at, 2003) Inhibitory effects of EGCG in several signal transduction pathways have been proposed like the p38, JNK and ERK pathways (Katiyar et al, 2001). Studies have also shown that EGCG prevents the binding of EGF to EGFR, preventing the induction of several EGF pathways including Ras associated ones (Masuda et al, 2001; Masuda et al, 2002). Due to its inhibitory effects on AP-1 along with its direct effects, EGCG plays a very important role in suppressing MMP activity and production (Dong et al, 1997).

Chapter 2. Materials and Methods

Cell Cultures

The IBC-10a cell strain was isolated from the right peripheral zone of a prostate gland with Gleason score 6 tumors. Cells were immortalized by LXSN-hTERT retroviral transfection (courtesy of John Rhim, Center for Prostate Disease Research, USUHS, Bethesda, MD) using methods previously described (Miki et al, 2007). IBC-10a cells were found to express CK5, CK18, p63 and PTEN and have been categorized as intermediate basal cells (Goodyear et al, 2009). Cells were maintained in serum free complete keratinocytes media (cKm) containing EGF, pituitary extract and 1% penicillin/ streptomycin (Invitrogen Inc., Carlsbad, CA). PCa-20a cells were isolated from human Gleason score 7 prostate tumors. They were found to express CK5, CK18, p63 and PTEN and have been categorized as intermediate basal cells (unpublished data). Cells were maintained in serum free complete keratinocytes media (cKm) which contains EGF,

pituitary extract and 1% penicillin/ streptomycin (Invitrogen Inc., Carlsbad, CA). PC3 cells, called PC3ML2 isolated based on their ability to metastasize to the lumbar vertebrae (Wang and Stearns, 1999), were used in some of the experiments. PC3ML2 cells were maintained in DMEM with 10% fetal bovine serum (FBS) according to previously described protocols of ATCC (Amer. Tissue Culture Consortium, Bethesda, MD). All the above cell cultures were maintained at 5% CO_2 and 37°C.

Stable over-expression of Ras constructs

Ras mutant constructs were stably overexpressed in both IBC-10a and PCa–20a cells using a pBABE-puro retroviral vector (pBABE:RasV12, pBABE:RasV12C40, pBABE:RasV12G37 and pBABE:RasV12S35 were kind gifts from Dr. Christian Sell, Drexel University College of Medicine). Both retroviral production and maintenance of transduced cells was carried out according to methods described by the Nolan lab (Wolkowicz, 2004). Briefly, viral particles were isolated from supernatants of transiently transfected PhNxA cells. IBC-10a and PCa-20a cells were incubated with retroviral supernatants in 10 ug/ml polybrene overnight and stable selection of Ras over expressing cells were obtained by culturing cells in the presence of puromycin (5 µg/ml; Sigma-Aldrich, St Louis, MO) for one week. The empty vector (pBABE-puro-Empty; was a gift from Dr. Christian Sell, Drexel University College of Medicine) was used as a control (The above transfections were performed by Michael Amatangelo).

Cell and media treatment with growth factors and EGCG

Primary intermediate basal cells or primary prostate cancer cells (IBC-10a and

PCa-20a) were obtained from the stock flask. For the E+T studies, the cells were pretreated overnight with Km (minimal media) after which they are treated with EGF (10ng/ml), TGFβ1 (10ng/ml) or E+T (EGF and TGFβ1, 10ng/ml each). (EGF and TGFβ1 concentrations of 5ng/ml and 10ng/ml were used interchangeably due to their similar effects on MMP2 and MMP9 production). The cells were treated for 7 or 9 days and the media was changed every 3 days over this period. The media was collected from the cells after an overnight treatment with fresh media (with or without ligands). For the EGCG cell treatment studies, PC3ML2 cell and IBC-10a cells were treated with increasing concentrations of EGCG. The IBC-10a cells were treated with E+T for 6 days followed by an overnight treatment of EGCG (10uM or 30uM) at 37°C. The PC3ML2 cells are grown in DMEM with 10% Fetal Bovine Serum and are directly treated overnight with EGCG (10uM, 30uM, 50uM and 100uM) at 37°C. For the media treatment with EGCG, the media from untreated PC3ML2 cells was collected. EGCG at different concentrations was added to the media and incubated overnight at 37°C. To identify the effects of EGCG on MMP gelatinase activity, zymograms containing MMPs were incubated with EGCG overnight. After running the protein samples on the gels, they were incubated overnight in separate containers, with and without EGCG at 100uM. After the incubation period, the gels were stained using Coomassie blue.

Protein extraction

After every experiment, the media from the cells was collected following an overnight treatment of the respective experiment. Cell lysis was performed by using 250ul of lysis buffer (RIPA buffer, 1% protease inhibitor cocktail and 1% Na_3VO_4).

The collected media were concentrated using Amicon Ultra- 15 centrifugal filter devices (Millipore Corp., Bedford, MA). The filters were prewashed with PBS followed by centrifugation at 4°C and 5000 rpm for 10 minutes. Alternatively, dialysis bags (SpectroPor membrane, MWCO:10,000, Spectrum Labs) were used, where the media was dehydrated using and Polyethylene Glycol (molecular weight: 20,000). Both these methods resulted in 250- 500ul of protein concentrate. The protein concentration of the cell lysates and concentrated media were calculated using the Bradford assay at 595nm. A 1mg/ml BSA solution was used as the standard for the assay. A standard curve was determined in order to obtain the relative protein concentration of the collected samples.

Gelatin Zymography

Gelatin zymography was performed using 10% gels containing Gelatin A (2mg/ml). The gels were placed in a gel electrophoresis chamber.1x running buffer (Tris HCL, Glycine and SDS) was used as an electrolyte. 0.01-1.5ug of the samples were loaded which were mixed with 1x sample buffer. The samples were run at 100V for approximately 1.5 hours. The gels were removed from the stacks and placed in the incubation buffer (Tris HCl, $CaCl_2$, NaCl, Brij35) for an overnight incubation. The gels were stained using 0.2% Coomassie Blue and then destained using the destaining solution (25:65:10 of Methanol, Acetic acid and water respectively). They were analyzed and imaged using the ChemiDox XRS Gel Documentation system (Bio Rad Laboratories, Inc., Hercules, CA)

Quantification of Zymography data

The zymography data was quantified using Image J software. The digested bands on the zymogram (image type- TIFF) were selected using the rectangular tool. As zymograms are binary images, default Image J settings were used which assumed white objects on a black background. [100] The area values obtained from the software for the experimental samples were normalized against the negative control group (Km or untreated) for all the experiments.

Statistical Analysis

Results from the first two specific aims were statistically analyzed using one way ANOVA comparison (SPSS) of the treated samples ($p<0.0256$). Each treatment group was further compared to the negative control via one sample t- test (SPSS) ($p<0.0256$). The graphs obtained from the quantified data represented the mean $\pm$ standard deviation. The differences between treated groups were analyzed using Tukey post hoc test ($p<0.0256$). The statistical significance of the third specific aim was analyzed using a one sample t- test (SPSS). One way ANOVA was not performed on this data as only two treated groups were used in this section. Further, the differences between treated groups were indicated with an asterix on the graphs. Homogenous subsets are denoted by letters (A-C), where groups of one subset are denoted by the same letter.

Chapter 3. Design component

Problem identification

Prostate cancer is one of the most common forms of cancer and is a second leading cause of cancer death in males. (Carter and Coffey, 2006) Especially affecting the

older population, the progressed forms of this disease metastasizes to various organs, most commonly to the bone. (Odero-Marah, 2008) The migration of these malignant prostate cells to the bone and other areas is poorly understood. The epithelial to mesenchymal transition (EMT) is a critical step in converting a benign tumor to a malignant one (Polyak and Weinberg, 2009). It has been recently identified that ligand binding is one of the ways prostate cancer cells progress into EMT phenotype and TGFβ1 plays a very important role in this regard (Deckers et al, 2006). This cytokine plays a dual role- it is tumor suppressor in the initial stages of cancer but has been found to assist in promoting tumor malignancy in the latter stages (Bierie and Moses, 2006). TGFβ1 has canonical and non canonical pathways that enable it to induce tumor invasive activities in cancers (Zhang et al, 2009). Its cross talk with the EGF has been associated with increased invasive capacity in many cancer types (Denys et al, 2008; Odero-Marah et al, 2008). This study aims to examine the synergistic effect of EGF and TGFβ1 on MMP2 and MMP9 secretion by primary prostate cancer cells and determine if an intact Ras is required for E+T induced EMT in these cells.

Currently, one primary goal of cancer research is to identify agents that inhibit EMT, indirectly blocking metastasis. It was recently observed that green tea drinkers have a lower incidence of prostate cancer compared to the rest of the population, suggesting anti- cancer effects of green tea ingredients. EGCG is one of the most prominent anti oxidants in green tea that contributes to this effect (Fujiki et al, 2002; Valcic et al, 1999). This molecule has enormous potential in drug therapies and other forms of cancer prevention techniques primarily because of its low toxicity in healthy cells. Although androgen deprivation is the main form of therapy in prostate cancers, a

few chemotherapeutics that block the EMT exist, like Docetaxel, Prednisone and Mitoxantrone. Recent studies have shown that these drugs are more effective when used in combination with each other. (Tannock et al, 2004) EGCG can thus potentiate other chemotherapeutic agents and increase the efficacy of the treatment with fewer side effects. (Stuart and Rhonda, 2008) The combination of raloxifene and EGCG suppresses growth and induces apoptosis in MDA-MB-231 cells. Therefore, an added goal of this study is to determine if EGCG can inhibit EGF and TGFβ1 induced EMT in primary prostate cancer cell lines.

Goal

We aim to deduce the specific ligands that can induce EMT via MMP production in primary prostate cancer cell lines. An overlapping goal is to examine whether green tea extract (EGCG) can inhibit MMP production during EMT. The long term goal of this study is to design a therapeutic approach for prostate cancer using EGCG.

Hypothesis

Growth factors, EGF and TGFβ1, synergistically drive prostate epithelial cancer cells towards an EMT phenotype by increasing the MMP2/9 production in them and thus increasing their invasive capacity. EGCG has inhibitory effects on MMP production and thus blocks EMT in cancer cells.

Design objectives/ Specific Aims

Aim1: To evaluate the effects of EGF and TGFβ1 on the MMP2 and MMP9 secretion in primary prostate cancer cells.

Aim2: To determine the importance of an intact Ras signaling pathway for E+T induced MMP2 and MMP9 secretion.

Aim3: To determine the inhibitory effects of EGCG (green tea extract) on the E+T induced MMP secretion in primary prostate cancer cells and in PC3ML2 cells.

(a) Aim1

To evaluate the effect of EGF and TGFβ1 on the MMP2 and MMP9 secretion in primary prostate cancer cells (IBC-10a and PCa-20a).

(i) Rationale:

Studies indicate that androgen independent tumors survive and progress with the help of growth factors like EGF and TGFβ found in the tumor microenvironment under the pressure of androgen deprivation in some patients undergoing hormone ablation treatment or other unknown reasons. (Klein et al, 1997) It was initially thought that mutations and the presence of oncogenes were necessary for the initiation and progression of tumors. Most commonly the H-Ras mutation in prostate cancer has been associated with cancer progression, particularly with its conjunctive effort with TGF β1. However, more recent studies have shown the presence of growth factors like EGF and TGF β1 in the tumor stroma and have been associated with the initiation and progression of several steps in these cancers.(Hazelbag et al, 2002) Preliminary results have shown that the induction of an EMT phenotype (high Vimentin expression and low E-cadherin expression) in human prostate cancer cells co-treated with EGF and TGFβ1. (Amatangelo, personnel communication) Our goal is to determine if the combined EGF and TGFβ1 treatment can increase MMP2 and MMP9 production in primary prostate

cancer cells with the help of gelatin zymography. High MMP2/9 secretion is associated with a rise in invasive capacity of cells. Invasiveness and increased migratory capacity are key features of metastatic cells that have undergone EMT and present a mesenchymal phenotype. (Liabakk et al, 1996)

(ii) Specific Aims:

To observe the effect of TGF β1 and EGF on MMP2/9 secretion in primary prostate cancer cells.

(iii) Experimental Design:

The media of IBC-10a and PCa-20a cells treated with Km, EGF, TGF β1 and E+T was concentrated and the MMP2/9 secretion in the media was measured using gelatin zymography. Additionally, the cell morphology of the cells after treatment was analyzed using phase contrast microscopy. Figure 6 displays a schematic of the experimental design for the first specific aim.

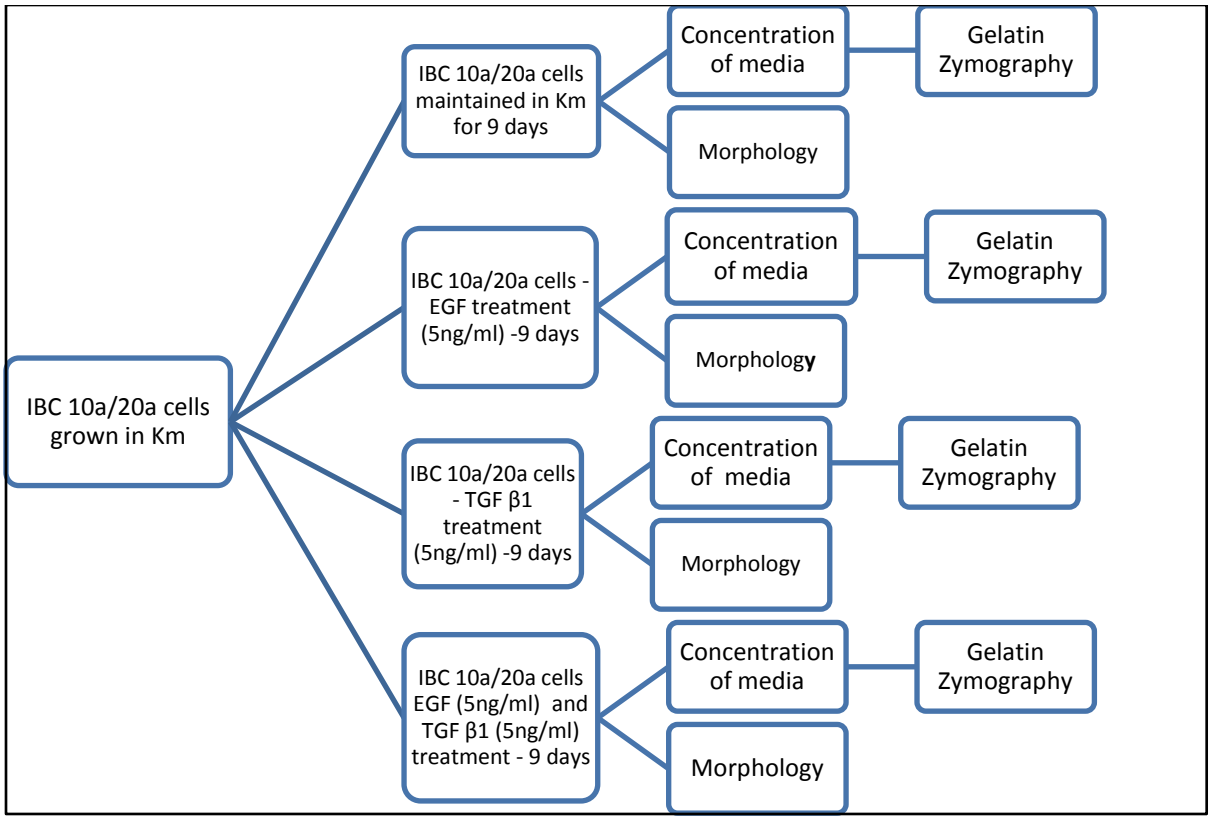


Figure 6: Schematic of experimental design for Specific Aim 1.IBC-10a/PC- 20a cells were grown in minimal media (Km) overnight before starting growth factor treatments. The treatments were maintained for 9 days with change of media every 3 days. The MMP2/9 secretion in the media collected from the cells was analyzed using gelatin zymography.

(iv) Criteria

Table 1: Design criteria for Specific Aim 1

Criterion	Rationale
1. Treat cells for 9 days with EGF and TGF β1.	Previous experiments have indicated that IBC-10a and PCa-20a cells take approximately, 9 days to acquire an EMT like phenotype. (Unpublished data) It was thus necessary to prevent highly confluent conditions in the cell

	plates and the cells were thus seeded at less than 25% confluence.
2. The MMP2/9 secretion in the EGF + TGF β1 treated samples should be higher than that in control samples (Km, EGF alone and TGF β1alone samples)	Earlier studies in prostate and other organs have shown the EMT phenotype being induced with a combined EGF and TGF β1 treatment of cells in vitro (Denys et al, 2008). Production of MMPs are characteristic of an EMT phenotype and they assist in increasing the migratory and invasive capacity of cancer cells.

(v) Constraints:

The main constraints for the first specific aim are as follows.

a. The seeding density of the cell dishes needs to be less than 25% in order to maintain the cell culture for a period of 9 days.
b. The treated cells release the MMPs into the media they are grown in and therefore the media needs to be used to analyze MMP2/9 secretion of the cells (treated and untreated).
c. The zymography needs to be performed with gelatin as the substrate as MMP2 and MMP9 are gelatinases.
d. Pretreatment of the collected media with amino phenyl mercuric acetate (APMA) is required to cleave the active band from the pro-enzyme. This step could not be performed due to time and monetary constraints.

(vi) Alternative solutions:

The above experiment identifies whether the EGF and TGF combination treatment can boost MMP2 and MMP9 levels in primary prostate cancer cells. Another area that needs to be analyzed is the regulatory mechanisms that support MMP production in these cells. Our results and other current studies have shown that there is a difference in the regulatory mechanisms of MMP2 and MMP9 primarily because of presence of distinct activators for the two MMPs (Egeblad and Werb, 2002). Therefore experiments measuring TIMP2, MMP14 and MMP3 levels post E+T treatment of primary prostate cancer cells will be beneficial in identifying the regulatory processes behind MMP activation. The levels of TIMPs in the cells can be measured using Western blotting or other protein expression assays like Enzyme Linked Immunosorbent Assay (ELISA).

Flourometric analysis is an alternative method to gelatin zymography for MMP2/9 secretion measurements. In such an assay, the MMP specific substrate contains a flourophore and a light absorbing quencher. These remain attached to an amino acid sequence recognized by the MMPs. The enzymatic activity of the MMPs cleaves the light absorbing quencher and this step allows the fluorescence to be observed. (Stack et al, 1989; Knight et al, 1992) The flourometric analysis helps analyze the active MMP2/9 secretion than measuring the total MMP2/9 secretion as seen in the protocol used in this study. However the main downside of using this assay is their low efficiency due to optical disturbances from the media and the presence of lipophilic substrates that leads to their low solubility in the samples. Beekman et al published a new flourometric protocol that used a water soluble flourogenic MMP substrate. Although this protocol increased the solubility of the substrate, the water soluble substrate they used was very general ad

cannot be used to identify specific MMPs, thus limiting the application of the protocol. (Beekman, 1996; Matyoshi et al, 1989)

(b) Aim2

To determine whether an intact Ras signaling pathway is essential for E+T induced EMT.

(i) Rationale:

It is well known that Ras acts downstream of the EGF pathway. Several studies have indicated the co-operation between TGF β1 and Ras in promoting an invasive phenotype is some cancer types. A study in breast cancer cells was able to show that Ras mutation along with TGF β1 treatment was necessary for the induction of an invasive phenotype. On further dissecting the Ras pathways using small molecule inhibitors, they were able to show that it was the MAPK pathway that TGF β1 cooperated with to induce an EMT phenotype which they measured using Vimentin and E-cadherin expression in the cells. (Janda et al, 2002) However, whether this co-operation between TGF β1 and the Ras pathway pertains to prostate cancer continues to be debate. The aim of the study is to initially identify the existence of the proposed co-operation between the two pathways as well as to identify the specific Ras pathway responsible for this interaction.

(ii) Specific Aims:

Aim 2a: To evaluate the effects of EGF and TGF β1 on MMP2 and MMP9 secretion in pBABE V12ras transfected IBC-10a on PCa-20a cells.

1) Experimental Design:

The media of pBABE.V12ras.IBC-10a cells treated with Km, EGF and TGF β1 was concentrated and the MMP2/9 secretion in the media was measured using gelatin

zymography. Additionally, the cell morphology of the cells after treatment was analyzed using phase contrast microscopy. Figure 7 below displays a schematic of the experimental design for the second aim.

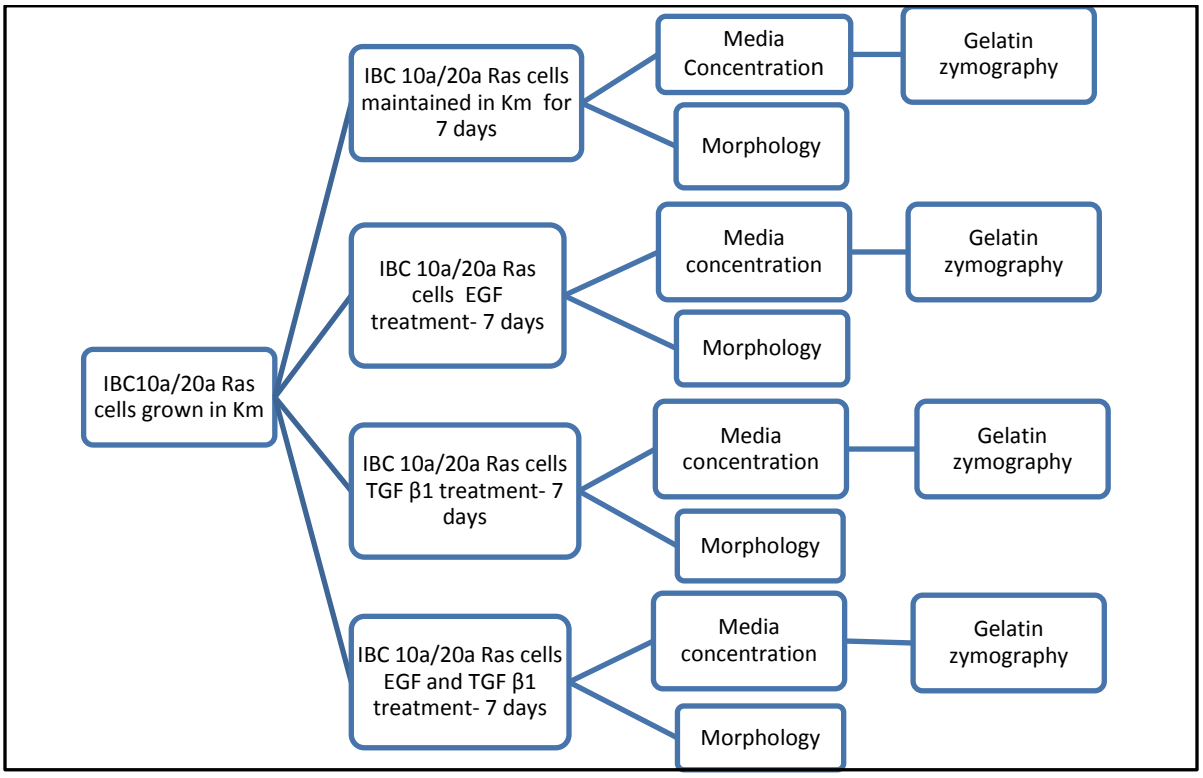


Figure 7: Schematic of experimental design for Specific Aim 2a. pBABE V12ras IBC-10a and pBABE V12ras PCa-20a cells were grown in minimal media (Km) overnight before starting growth factor treatments. The treatments were maintained for 9 days with change of media every 3 days at a concentration of 5ng/ml of EGF and TGFβ1. The MMP2/9 secretion in the media collected from the cells was analyzed using gelatin zymography

2) Criteria:

Table 2: Design criteria for Specific Aim 2a.

Criterion	Rationale
1. The basal levels of MMP2/9 in pBABE.V12ras.IBC-10a cells are	Ras transformation has been shown to increase the invasive capacity of

more than that in IBC-10a parent cells.	cancer cells by increasing their MMP activities. Several studies have shown the induction of MMP expression in Ras transformed cells via the NF-KB pathway which was also able to simultaneously decrease the TIMP levels in the cells. (Yang et al, 2001). The induction of MMPs via Ras pathways are also found to be assisted with the help of transcription factors like AP-1 and Ets-1 (Westermarck et al, 2001).
2. The MMP2/9 activities in the TGF β1 Ras transformed IBC cells is similar to the MMP2/9 activities of Ras cells treated with a combination of EGF and TGF β1.	Ras transformed cells have been treated with TGF β1 in several cancer studies and the results have indicated an increase in invasive activity in these cells post treatment with this growth factor. Several studies have directed this co-operation between Ras and TGF β1 to the induction of the MAPK pathway. (Park et al, 2000; Janda et al, 2002) An increase in invasive activity in vitro and indications of metastasis in vivo suggests a possible increase in MMPs which generally assists these processes.

3) Constraints:

a. The Ras cells in this experiment need to be treated for a period of seven days for the cells to attain an EMT phenotype.

b. The seeding density of the cells on the 10cm dishes should be less than 25% to prevent highly confluent conditions.

c. The media from treated samples needs to be collected on day seven after the media is replaced in the dishes on day six to measure the MMP release of the cells overnight and not the cumulative MMP released over several days.

d. The media samples can be analyzed using gelatin zymography to measure MMP2 and MMP9 activities as they are gelatinases.

e. In order to obtain quantifiable images, only 0.05ug of protein could be loaded per well.

f. Effective treatment range for EGF and TGF β1 needs to be between 1ng/ml and 10ng/ml.

g. Pretreatment of the collected media with APMA is required to cleave the active band from the pro-enzyme. This step could not be performed due to time and monetary constraints.

4) Alternative solutions:

Flourometric analysis is an alternative method to gelatin zymography for MMP2/9 secretion measurements. In such an assay, the MMP specific substrate contains a flourophore and a light absorbing quencher. These remain attached to an amino acid sequence recognized by the MMPs. The enzymatic activity of the MMPs cleaves the light absorbing quencher and this step allows the fluorescence to be observed. (Stack et al, 1989; Knight et al, 1992) The flourometric analysis helps analyze the active MMP2/9

secretion than measuring the total MMP2/9 secretion as seen in the protocol used in this study. However the main downside of using this assay is their low efficiency due to optical disturbances from the media and the presence of lipophilic substrates that leads to their low solubility in the samples. Beekman et al published a new flourometric protocol that used a water soluble flourogenic MMP substrate. Although this protocol increased the solubility of the substrate, the water soluble substrate they used was very general ad cannot be used to identify specific MMPs, thus limiting the application of the protocol. (Beekman, 1996; Matyoshi et al, 1989)

As MMPs are indicative of the migratory capacity of the cells, migration and invasion assays can be used in Ras cells to compare the migratory capacities with and without growth factor treatments. In these methods, the cells to be analyzed are seeded on the top chamber of the two chamber assay system which is part of the cell culture inserts. After the incubation period the cells migrated into the lower chamber is measured and this number is correlated to the migration and invasive capability of the cells. (Brand et al, 2005) However this is not a quantitative method to measure MMP activity and restricts the analysis to the identification of the number of migrated cells. Additionally, this method is more a measure of invasiveness which is indirect measurement of MMP2/9 secretion compared to gelatin zymography.

There are several methods to measure MMP proteins levels in the media. ELISA and Western blotting are alternative methods to measure the MMP2/9 production in the media quantitatively and qualitatively respectively. But with respect to this study, this method has several downfalls too. Like the migration assays, the ELISA and Western blotting cannot be used to measure the enzymatic activity of MMPs. These tools can only

be used to measure the MMP2/9 protein levels in the media. Thus gelatin zymography will be used for the measurement of MMP2/9 secretion.

Aim2-b: To determine the Ras pathway that cooperates with TGF β1 to increase MMP2/9 secretion.

5) Experimental Design:

In order to identify the Ras pathway that interacts with TGF β1 to drive cells to an invasive phenotype, Ras mutants with specific point mutations at V12, C40, G37 and S35 were obtained, which activated the PI3K, RalGDS and MEK pathways respectively. While activating the specific pathways, all other Ras pathways get inhibited in these mutant cells. They do so by binding preferentially to one effector but failing to bind to the other two. (Rodriguez-Viciana et al, 1997) These mutants were treated with TGF β1 (10ng/ml) for 7 days. The media from the treated cells was obtained which was analyzed for MMP2/9 secretion using Gelatin zymography. Figure 8 displays a schematic representation of the experimental design for Specific Aim 2b.

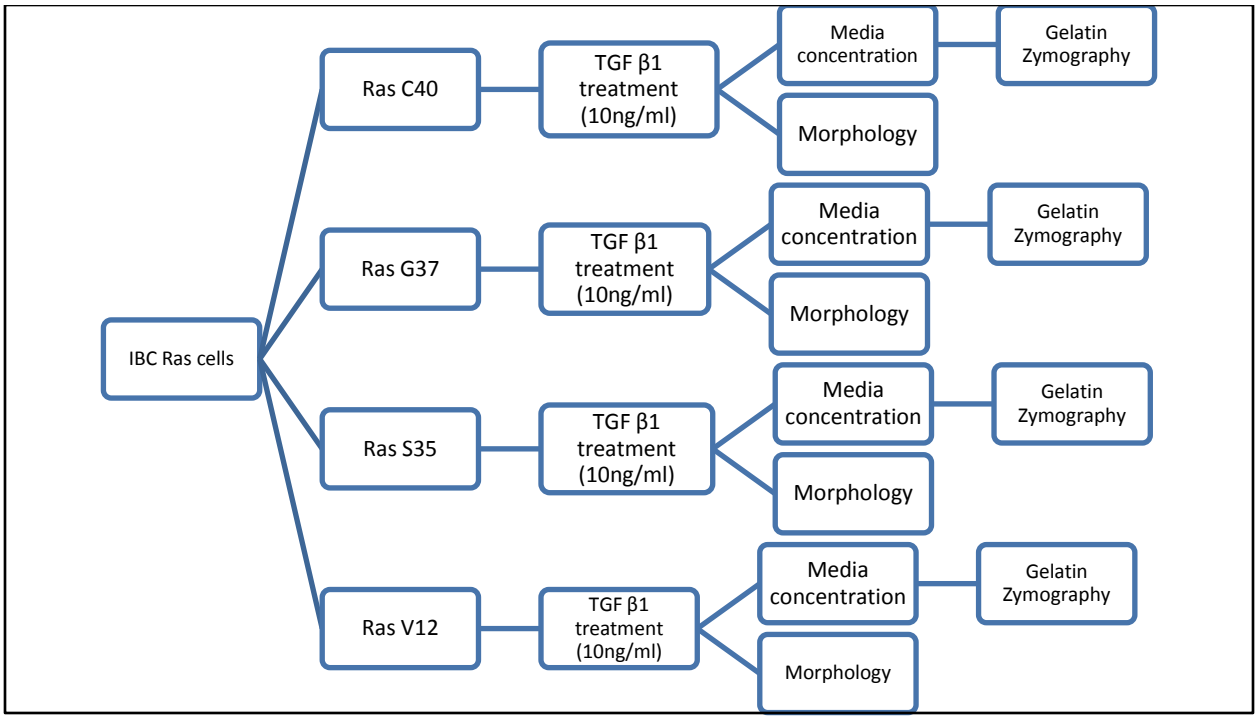


Figure 8: Schematic of experimental design for Specific Aim 2b. pBABE Ras V12 IBC-10a and pBABE RasV12 PCa-20a Ras mutant cells were grown in minimal media (Km) overnight before starting growth factor treatments. The treatments were maintained for 7 days with change of media every 3 days. The media collected from the treated cells was used for gelatin zymography to analyze MMP gelatinase activities.

6) Criteria:

Table 3: Design Criteria for Specific Aim 2b

Criterion	Rationale
1. The modified Ras cells should be able to activate only one Ras pathway and disable the other pathways.	The RasV12 cells were mutated specifically at amino acids C40, G37 and S35 in order to preferentially activate the PI3K, RalGDS and the MEK pathways respectively. In order to identify the pathway that cooperates with TGF β1 in inducing invasive properties in Ras cells, it is necessary

	to activate the Ras pathways we are interested in analyzing individually (Rodriguez-Viciana et al, 1997). In this way, only the activated Ras pathway possesses the ability to interact with TGF β1. The Ras mutant cells that show highest MMP2 and MMP9 activities when compared against other Ras mutants will be the one responsible for the increased invasive activity in the cells as displayed by the MMP2/9 secretion.
2. The MMP2/9 activities in one of the Ras mutants should exceed the MMP2/9 activities in the other mutants and similar to MMP2/9 activities in the normal Ras control cells.	The most invasive Ras mutant working in conjunction with TGF β1 will be expected to produce comparatively higher levels of MMP2 and MMP9. Earlier studies have shown that Ras cells possess a higher invasive potential post TGF β1 treatment (Oft et al, 1996). Therefore, the Ras mutant responsible for the invasive phenotype of TGF β1 treated Ras cells will be measured using MMP2 and MMP9 activities.

7) Constraints:

a. The Ras cells in this experiment need to be treated for a period of seven days.

b. The seeding density of the cells on the 10cm dishes should be less than 25% to prevent highly confluent conditions.

c. The media from treated samples can needs to be collected on day seven after the media is replaced in the dishes on day six to measure the MMP release of the cells overnight and not the cumulative MMP released over several days.

d. The media samples can be analyzed using gelatin zymography to measure MMP2 and MMP9 activities as they are gelatinases.

e. In order to obtain quantifiable images, only 0.05ug of protein could be loaded per well.

f. Treatment range for EGF and TGF β1 needs to be between 1ng/ml and 10ng/ml.

8) Alternative solutions:

An alternative method to identify the key Ras pathway that cooperate with TGF β1 is to use small molecule inhibitors of the Ras pathway namely LY21, LY29 and PD098 that have the ability to inhibit the TGF beta pathway, Akt pathway and the MEK pathway respectively. These drugs inhibit effectors of Ras and thus inhibit their activation. LY29 inhibits PI3K by binding to the ATP- binding site of PI3K enzyme. This action usually leads to cell apoptosis in cancer cells induced by blocking the PI3K-Akt nexus (Poh and Pervaiz, 2005). The Ras cells that fail to demonstrate a relatively high MMP2 and MMP9 activities after the inhibitor treatment along with TGF β1 will direct us to the right pathway that is necessary for MMP2 and MMP9 activation, which will also suggest the pathway that cooperates with TGF β1 to increase the invasive capacity of cancer cells.

(c) Aim3

To determine the effect of EGCG (green tea extract) on MMP2 and MMP9 secretion in malignant (PC3ML2) and pre-malignant (IBC-10a, PCa-20a) cell lines.

(i) Rationale:

Significant evidence has been provided in the past to support the anti- tumorigenic effects of EGCG in rodents as well as in humans through clinical trials. Epidemiological studies recently performed have been able to show that this green tea extract had significant anti cancer effects in prostate cancer, a result found in several other forms of cancer. (Bushman, 1998; Nelson, 2004; Jian et al, 2004; Gupta et al, 2001) Although several studies have shown the inhibitory effects of this potential drug in cancer models, the mechanism of action of the drug and its effects on MMPs remain unanswered. (Shannon et al, 2009) Thus, it is clear that more investigation is required to identify the effects of EGCG on the inhibition of prostate cancer metastasis. In this study we aim to identify the effects of EGCG on the MMP activities in malignant and pre- malignant prostate cell types.

(ii) Specific Aims

Aim3a: To identify the concentration of EGCG that can effectively inhibit MMP2/9 in PC3ML2 and IBC cells.

1) Rationale:

The effective concentration of EGCG to inhibit invasive activity in cells varies from one study to another and these values change based on the studied cell types. In a prostate cancer EGCG study, a concentration of 1uM was able to inhibit cell growth however they did not measure MMP2/9 secretion in these cells (Paschka et al, 1998). Another study showed the inhibition of MMP2 and MMP9 with EGCG at concentrations ranging

between of 0.1nM to 100uM (Garbisa et al, 2001). Therefore it was necessary to identify the effective concentration of EGCG in minimizing MMP activities in our prostate cells.

2) Experimental Design:

In order to observe the effects of EGCG on PC3ML2 and IBC cells it was necessary to identify the dose of EGCG that can effectively inhibit MMP2 /9 in both cell types. Therefore, a concentration study was performed on the cells. The primary difference between the PC3ML2 cells and the IBC cells is their degrees of malignancies; PC3ML2 cells are invasive and proliferate at a high rate whereas IBC cells are premalignant and less invasive. This difference makes them attain EMT at different rates and conditions. PC3ML2 cells do not need an external stimulus to attain an EMT phenotype but the IBC cells need to be pre- treated with a combination of EGF and TGF β1 to obtain an EMT phenotype. The following diagrams provide a schematic of the experimental designs used for each of these cell types. Fig 9 displays the experimental setup for PC3ML2 cells (malignant) and the effect of EGCG on these cells was tested using higher concentrations of EGCG ranging from 0uM to 100uM.

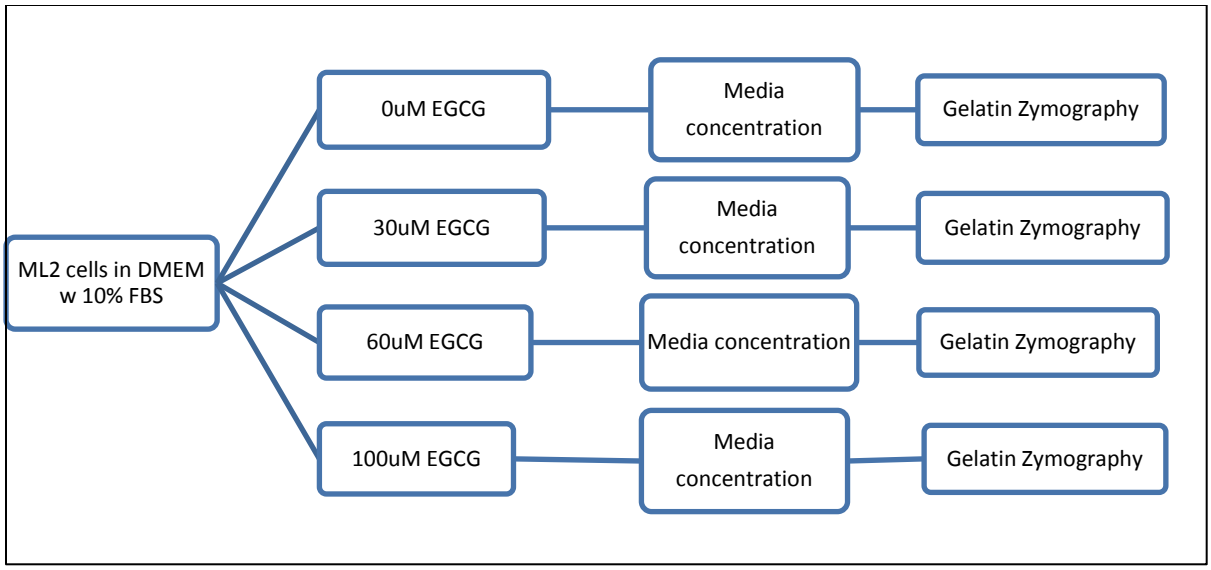


Figure 9: Schematic of experimental design for Specific Aim 3a. PC3ML2 cells were grown DMEM with 10% FBS before starting EGCG treatments. The cells were treated overnight . The MMP2/9 secretion in the media collected from the cells was analyzed using gelatin zymography.

The experimental design for the IBC cells is displayed in the schematic below (Figure 10). These premalignant cells were treated with EGCG concentrations falling under a smaller range between 0uM and 60uM.

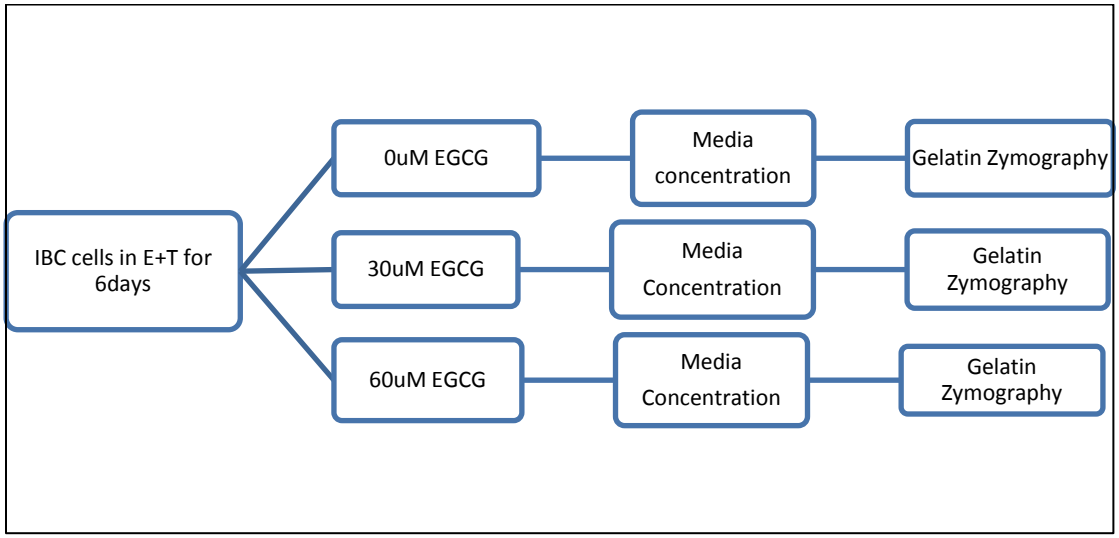


Figure 10: Schematic of experimental design for Specific Aim 3b. IBC cells were grown E+T for 6 days before starting EGCG treatments. The cells were treated with EGCG overnight.

The MMP2/9 secretion in the media collected from the cells was analyzed using gelatin zymography.

In each case the media of the cells treated with the "drug" was collected, concentrated and used for gelatin zymographic analysis.

3) Criteria:

Table 4: Design Criteria for Specific Aim 3a

Criterion	Rationale
1. The MMP2 and MMP9 activities of cells should decrease with increasing concentrations of EGCG.	The role of EGCG as a drug has not been entirely been established till date. However, studies in the past have shown its inhibitory effects on cancer cells including prostate cancer cells. (Garbisa et al, 2000) In particular, its effects on MMPs have been examined where EGCG was found to inhibit MMP2 and MMP9 activities (Cheng et al, 2003).

4) Constraints:

a. The malignant and premalignant prostate cells need to be treated with cells without reaching toxic levels of the drug that causes apoptosis of the cells.

b. The media of treated cells should be collected before the cells undergo apoptosis.

c. The EGCG needs to be used within 15-20 minutes after dissolution to prevent degradation due to the short half life of the molecule. (Rahman et al, 2006)

5) Alternative solutions:

The Biotrak activity assay system is an alternative to using gelatin zymography for measuring the inhibition of gelatinases by EGCG. This method uses microtiter wells coated with anti MMP antibodies. The conditioned medium is added to the wells and incubated overnight at 4°C. The MMPs are activated using APMA followed by an incubation period at 37°C in the presence or absence of EGCG. The intensity of the color obtained after the gelatinase digestion is measured at 405nm. However, this method could not be used due to lack of access to the Biotrak assay system and budgetary constraints.

Aim3b: To observe the effect of EGCG directly on MMP2/9 by treating the media obtained from untreated cells with EGCG.

6) Rationale:

Most EGCG studies performed in the past have been unsuccessful in identifying the mechanism of action of this molecule and its interaction with MMPs. A few of these studies indicate the inhibition of MMP2/9 secretion in these cells via direct binding of EGCG to the MMPs leading to its inactivation. Garbisa et al showed that EGCG was able to reduce cell proliferation at a concentration of 4uM and 0.08uM which suggests the presence of its multiple cellular targets. It is speculated that the inhibition of MMPs by EGCG is due to zinc chelation, but Garbisa and colleagues failed to see this effect in their experiments (Garbisa et al, 2001). Several questions about the molecular mechanisms of EGCG remain unanswered and treating the media collected from cells with EGCG could be potentially used to understand the physical interaction between EGCG and MMPs.

7) Experimental Design:

The media from PC3ML2 cells (malignant cells) were used for the following experiments due to the high levels of MMPs produced by these cells compared to the IBC cells. EGCG at 40uM and 60uM were added to media in different centrifuge tubes and incubated overnight at 37°C. In addition to the above procedure, gels containing MMPs were also incubated overnight at 37°C with EGCG to identify the direct effect of EGCG on inhibition of MMP gelatinase activity.

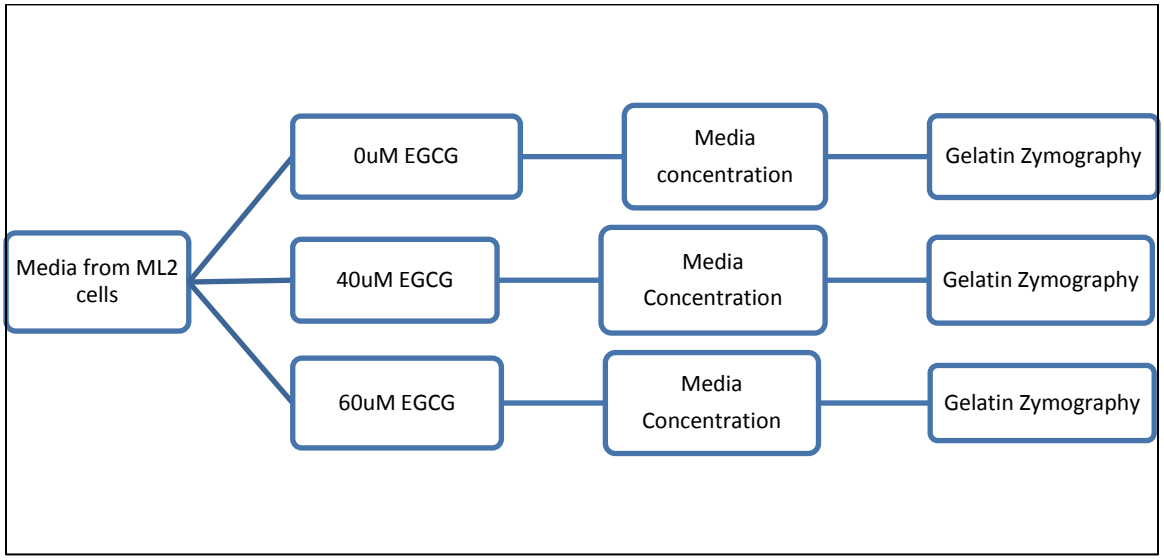


Figure 11: Schematic of experimental design for Specific Aim 3b. Media was collected from PC3ML2 cells. This media was treated with EGCG overnight. The MMP2/9 secretion in the media collected from the cells was analyzed using gelatin zymography.

8) Criteria:

Table 5: Design Criteria for Specific Aim 3b

Criterion	Rationale
1. The MMP2/9 secretion of the media treated with EGCG	A few studies in the past indicate that EGCG can inhibit MMP2/9 secretion

should be lower than the untreated (control) samples.	by binding directly to the MMPs and deactivating it (Garbisa et al, 2001). However this direct binding mechanism has not been fully proven in prostate cancer cells and therefore needs to be further investigated by treating media positive for MMPs with EGCG.

9) Constraints:

a. The media should be collected from PC3ML2 cells (malignant) or IBC cells that are treated with EGF and TGF for at least 7 days (premalignant) to ensure adequate basal MMP levels.

b. The media should be treated with EGCG within the range of 0 and 100uM of EGCG to compare results from the cell treatment study.

c. The EGCG needs to be used within 15-20 minutes after dissolution to prevent degradation due to the short half life of the molecule. (Rahman et al, 2006)

d. The media must be free of cell remnants to ensure that the results are purely obtained from the MMPs in the media and not the miscellany present in the cell pellets.

10) Alternative solutions:

The Biotrak assay system as mentioned earlier (specific aim 3a) could be used to detect the change in MMP2/9 secretion in the media before and after EGCG treatments.

Chapter 4. Results

1. The effect of EGF and TGF β1 on MMP2 and MMP9 secretion in primary prostate cancer cells

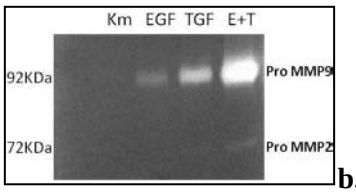
We aimed to observe the effects of TGF β1 and EGF on the MMP2 and MMP9 activities in primary prostate cancer cells. Preliminary studies were performed to identify the optimal EGF and TGFß1 concentrations for effective induction of MMPs in IBC cells (Refer to Appendix A). Accordingly, concentrations between 5ng/ml to 10ng/ml of both EGF and TGFß1 provided desired results. Thus most experiments used either 5ng/ml or 10ng/ml of EGF and TGFß1.

Measurement of MMP2/9 secretion using Gelatin Zymography

IBC-10a and PCa-20a cells (primary prostate cancer cells) were treated with Km (minimal media), EGF (5ng/ml), TGF β1 (5ng/ml) and EGF + TGF β1 (E+T) (5ng/ml each) for 9 days with a change of media every 3 days. The cells treated with Km were used as negative controls in order to detect the basal MMP2/9 levels in them. On day 8 the cells were given fresh media (with/ without ligands) which was collected on day 9. Figures 12a and 12b display the comparative effects of the ligands individually and in combination in IBC-10a and PCa-20a parent cells. ProMMP9 (latent form) activity increases with individual treatments of EGF and TGF β1 when compared to the negative control (cells treated with Km alone). However, there is a dramatic increase in the activities of both proMMP9 and the active MMP9 forms in cells treated with both ligands (E+T) when compared to rest of the controls (Figure 12).

The proMMP2 activity in PCa-20a parent cells followed a trend similar to the proMMP9 secretion, although the band intensity was lower in proMMP2 results. As expected the Km showed the least amount of activity, followed by EGF treated cells. The intensity and band width of proMMP2 bands increased significantly with TGF β1 treatment and the highest activity was observed in the E+T treatment (Figure 12).

In order to attain quantifiable results, it was necessary to obtain thinner bands. A loading of 0.01-0.05ug gave rise to quantifiable bands (thinner and distinct); however the active MMP9 bands were no more visible.

a. 

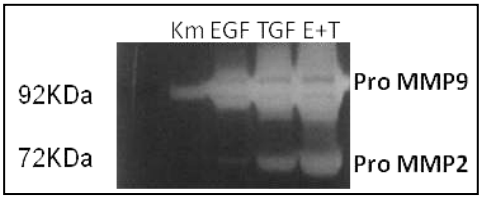
b. 

Figure 12: Gelatin Zymograms of PCa-20a cells. Above image shows zymograms of samples from PCa-20a cells treated with Km, EGF, TGFβ1 and EGF/TGFβ1 (5ng/ml each) for 9 days. The upper bands in the zymogram show Pro-MMP9 (92kDa) activity and lower bands show Pro-MMP2 activity (72kDa). The size and intensity of the bands represents the level of gelatinase activity of the MMPs. a. Each well was loaded with 0.05ug of the concentrated media collected at the end of the 9 day treatment. The media was changed every 3 days. b. Each well was loaded with 1.5ug of the concentrated media collected at the end of the 9 day treatment. The media was changed every 3 days.

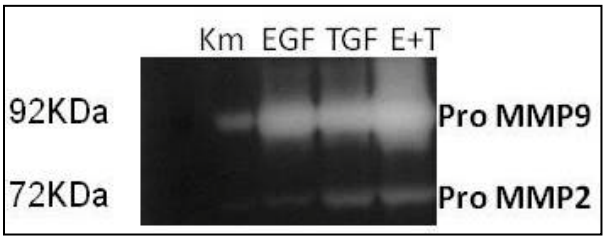


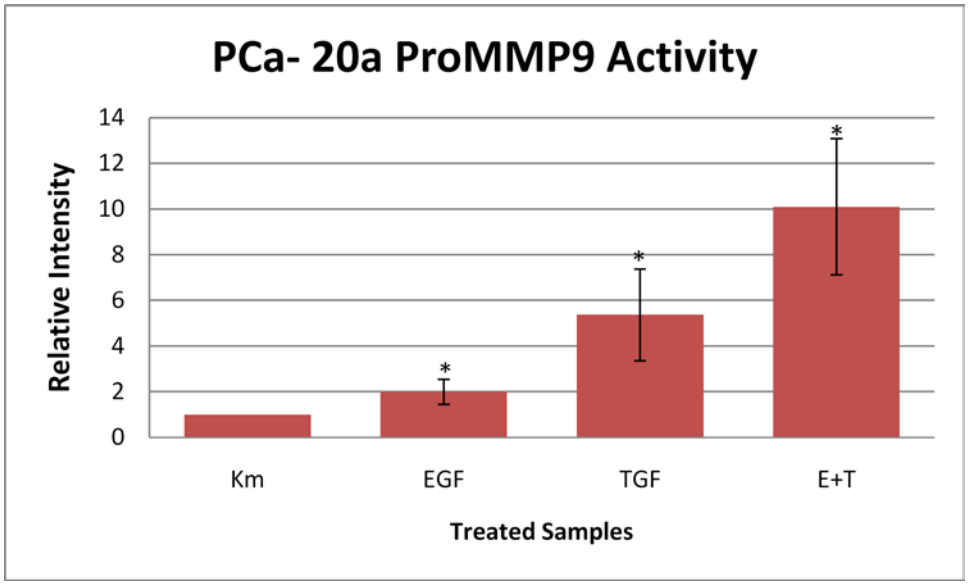
Figure 13: Gelatin zymogram of IBC-10a cells. The above image shows a zymogram of IBC-10a cells treated with Km, EGF, TGFβ1 and EGF/TGFβ1 (5ng/ml each) for 9 days. Each well was loaded with 1.5ug of the concentrated media collected at the end of the 9 day

treatment. The media was changed every 3 days. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 activity (72kDa). The active MMP9 bands are observed immediately below the proMMP bands for TGFβ1 and E+T treatments. The size and intensity of the bands represents the level of gelatinase activity of the MMPs.

The trend of increasing proMMP9 and proMMP2 activities with E+T treatment when compared to individual treatments and Km is maintained in IBC-10a parent cells (Figure 13). However, the active MMP9 bands are noticeable only in cells treated with E+T. The proMMP2 band activity in IBC-10a parent cells (Figure 13) increase drastically from nil activity in Km treated cells to a very faint band in EGF treated cells. There is a boost in MMP2 activity in TGF β1 treated cells when compared to the negative control, while maximum activity of proMMP2 is seen in the E+T treatment.

The graph in figure 14a shows proMMP9 secretion for PCa-20a parent cells treated with EGF, TGF β1 and E+T. Similar to the results observed in the zymograms, the graph shows maximum proMMP9 secretion in E+T treated samples with a relative intensity (RI) of 10 when compared to Km, EGF alone and TGF β1 alone with RIs of 1, 2 and 5 respectively. The active MMP9 bands could not be quantified with a protein loading ranging between 0.01ug and 0.05ug, but could be observed with higher protein loading (1ug). However, these gels were difficult to quantify due to the overlapping digestion bands among treated samples (Figure13). The quantified proMMP2 results are displayed in Figure 14b. The trend of increasing proMMP2 activity with E+T treatment is maintained. E+T treatment returned the highest RI of 16.34 when compared to Km, EGF and TGF β1 alone with RIs of 1, 3.4 and 11.2 respectively.

a.



b.

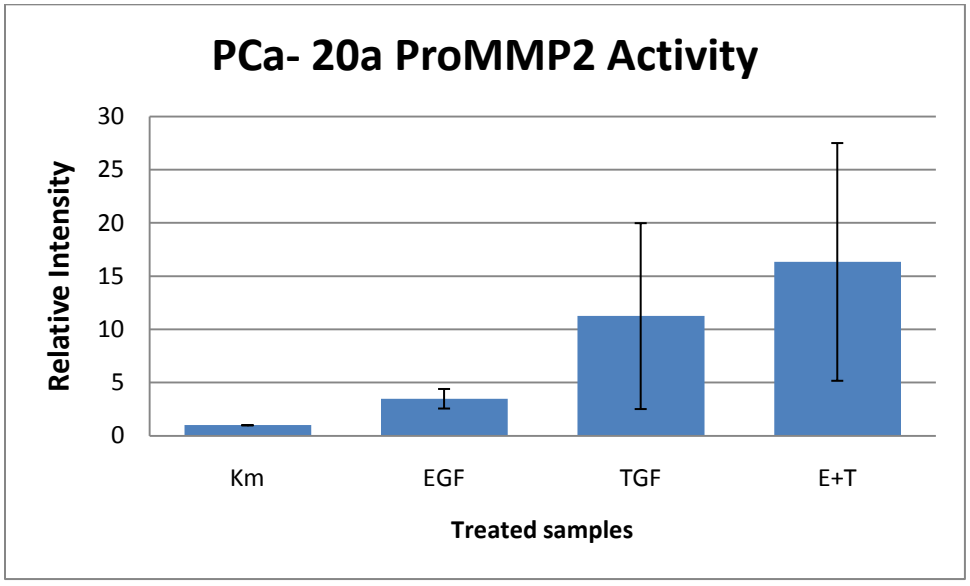


Figure 14: The graph comparing the proMMP9 secretion in untreated (Km) and treated (EGF, TGFβ1 and E+T) in PCa-20a parent cells. The above graph compares the proMMP9 secretion in untreated (Km) and treated (EGF, TGFβ1 and E+T) in PCa-20a parent cells. Data represents the mean ± SD of 3 replicate experiments. The error bars represent the standard deviation of the samples. The above activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was obtained by dividing the intensity value of treated samples by the Km activity value. a. proMMP9 secretion in Km samples indicates the basal levels in the PCa-20a parent cells. The E+T treated samples have maximum proMMP9 secretion when compared to samples treated with EGF and TGFβ1 alone. * indicates a significance ($p<0.0256$) in one way ANOVA with a Tukey post hoc (Groups are different from each other and are represented as A,B,C; A=EGF, B=TGF,C=E+T) b. This graph represents the proMMP2

comparison among the treated and control samples. The increasing trend of proMMP2 activity with E+T treatment is maintained.

Appearance of an EMT phenotype

The IBC-10a and PCa-20a parent cells were treated with 10ng/ml of EGF and TGF β1. After seven days of treatment with change of media every three days, phase contrast microscopy images were obtained. The E+T treatment induced an EMT phenotype in the parent cells as seen in Figure 15. Various morphological changes such as spindle shape morphology and lack of cell junctions were evident in the presence of E+T. The cells grown in Km were used as the negative control. They displayed cobblestoned morphology as expected of epithelial cells along with intact cell junctions. Additionally, Western blot analysis of E+T treatments revealed increased Vimentin and Fibronectin expression along with reduced E-cadherin and ß catenin. Similar results were obtained using Immunostaining. (Refer to Appendix C)

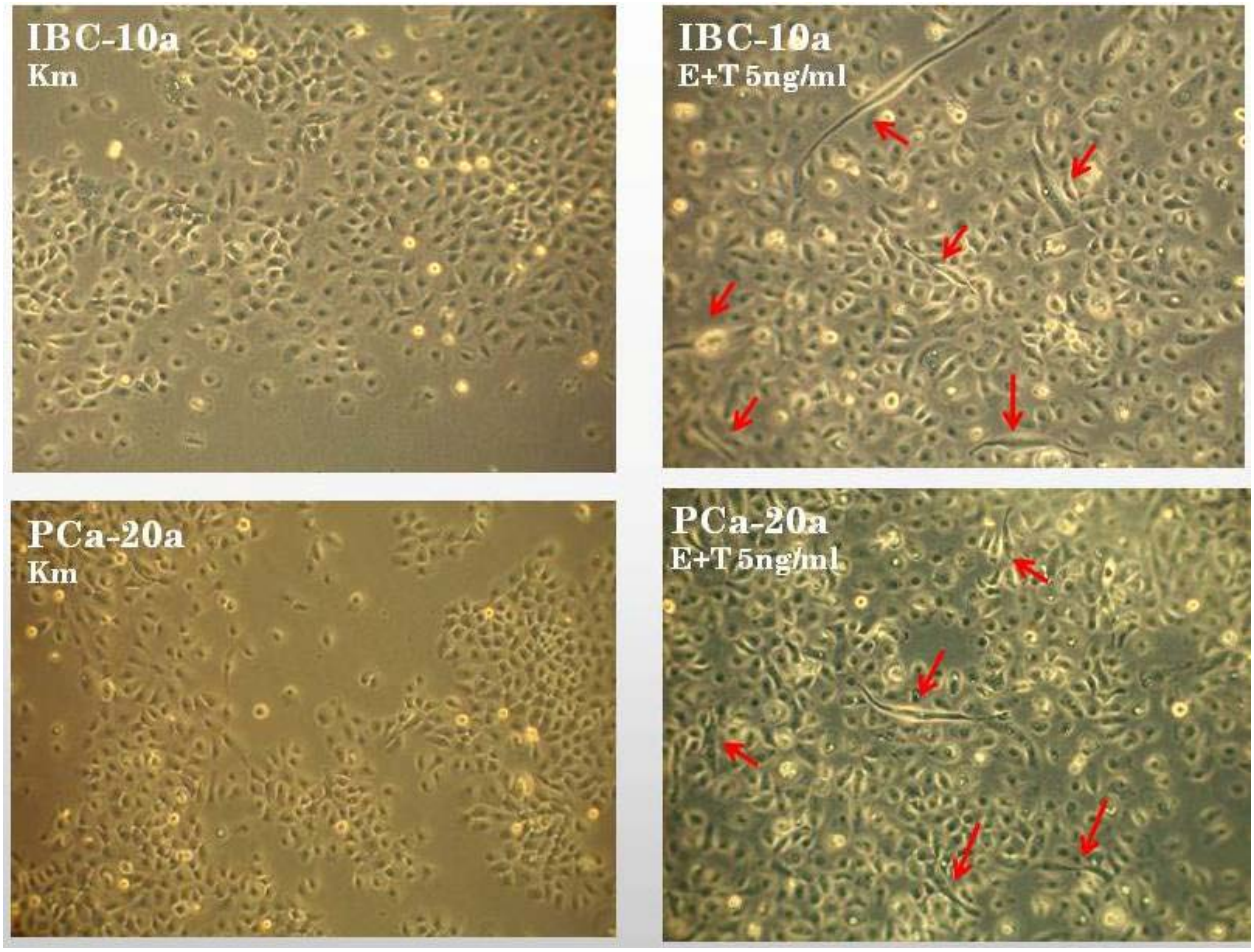


Figure 15: Gradual induction of an EMT phenotype in IBC-10a and PCa-20a parent cells treated with 5ng/ml of E+T. The red arrows in the bottom images indicate the presence of elongated, spindle like cells that exhibit a fibroblast like appearance. Cells grown in Km were used as the negative control, where they maintained cell junctions and have a cobblestone appearance of epithelial cells.

2. Intact Ras signaling required for E+T induced EMT in primary prostate cancer cells

Ras is a downstream effector of the EGF pathway and therefore we hypothesize that Ras overexpression can eliminate the need of EGF stimulation to assist TGF β1 in the induction of EMT and an invasive phenotype in cancer cells (Johnston, 2006). The effect of growth factors, EGF and TGFβ1, on the MMP2/9 production in Ras

overexpressing primary prostate cancer cells was determined by treating IBC-10a RasV12 (Figure 16) and PCa-20a RasV12 cells (Refer to Appendix B) with EGF, TGF β1 individually and in combination with each other (E+T) at a concentration of 5ng/ml.

Measurement of MMP2/9 secretion using Gelatin Zymography

The zymograms are displayed in figure 16 below. Km was used as the negative control in the zymograms to observe basal proMMP9 secretion. IBC-10a Ras cells treated with EGF alone did not significantly increase proMMP9 activities when compared to the TGF β1 alone and E+T treatments. Unlike results observed in parent cells, both TGF β1 and E+T combination treatment rendered very similar boost in proMMP9 secretion. The proMMP2 bands are low in band width and signal intensity for Km and EGF treated cells, but there is a drastic increase in this band intensity in cells treated with TGF β1 alone and with E+T. Similar results were obtained in the PCa-20a Ras cells (Refer to Appendix B). The active MMP2 and MMP9 bands are clearly visible only in cells treated with TGF β1 and E+T at higher protein loading of 1.5ug/ well (Figure 16b).

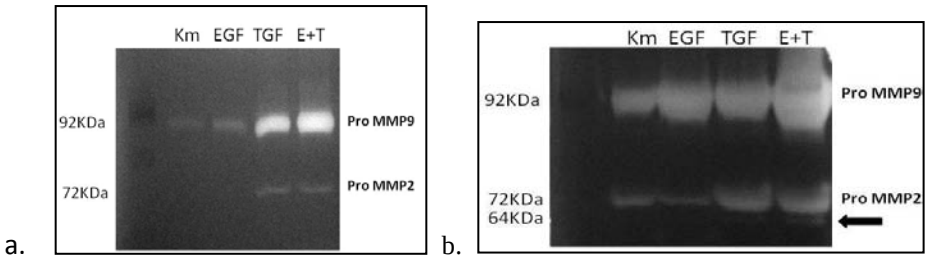


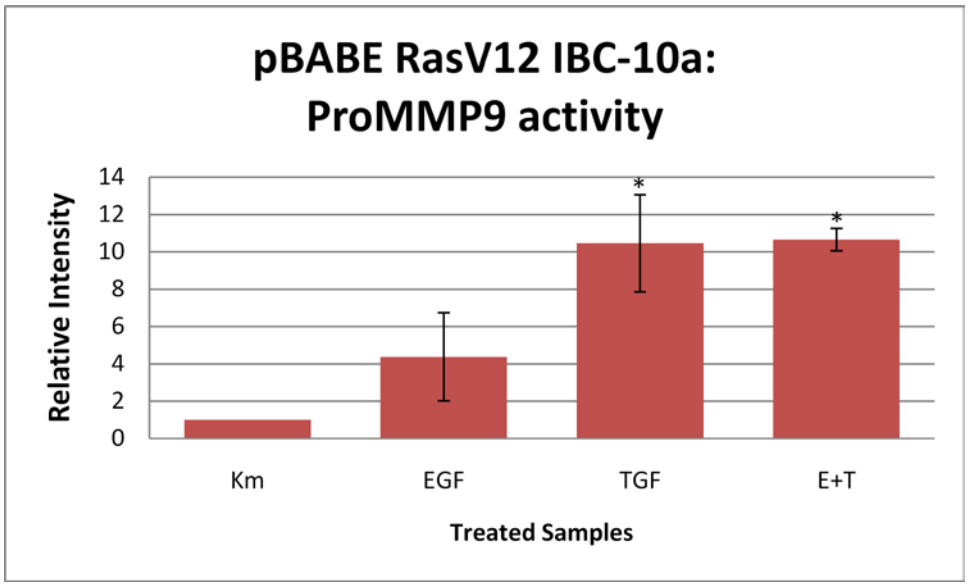
Figure 16: Gelatin Zymograms of IBC-10a Ras overexpressing cells treated with Km, EGF, TGFβ1 and EGF/TGFβ1. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. 5ng/ml of all growth factors were used for nine days. a. 0.05ug of protein was loaded per well in this zymogram. ProMMP2/9 activities of TGFβ1 and E+T yielded very similar results. The media was changed every 3 days. b. 1.5ug of protein was loaded per well in this zymogram. The arrows indicate the presence of active

MMP2/9 bands underneath proMMP2/9 bands respectively. ProMMP2 bands in TGFβ1 and E+T are very similar.

The graphs in figure 17 represent the quantitative analysis of the zymograms obtained using the Image J software. The graphical representation of the proMMP9 digestion data is very similar to the results obtained from zymographic analysis of the digestion bands. The TGF β1 and E+T treated samples show the highest proMMP9 secretion with an RI of 10.4 and 10.6 respectively (also very similar) when compared to the Km and EGF alone treated samples with RIs of 1 and 4.4 correspondingly (Figure 17a).

ProMMP2 gelatinase activity was very similar to the proMMP9 gelatinase results (Figure 17b). It was the highest in TGF β1 and the E+T treated samples, with RIs of 2.85 and 2.99 respectively compared to 1 and 1.04 of Km and EGF treatments respectively, thus showing the similarities in proMMP2 gelatinase activity in TGF β1 and E+T treatments.

a.



b.

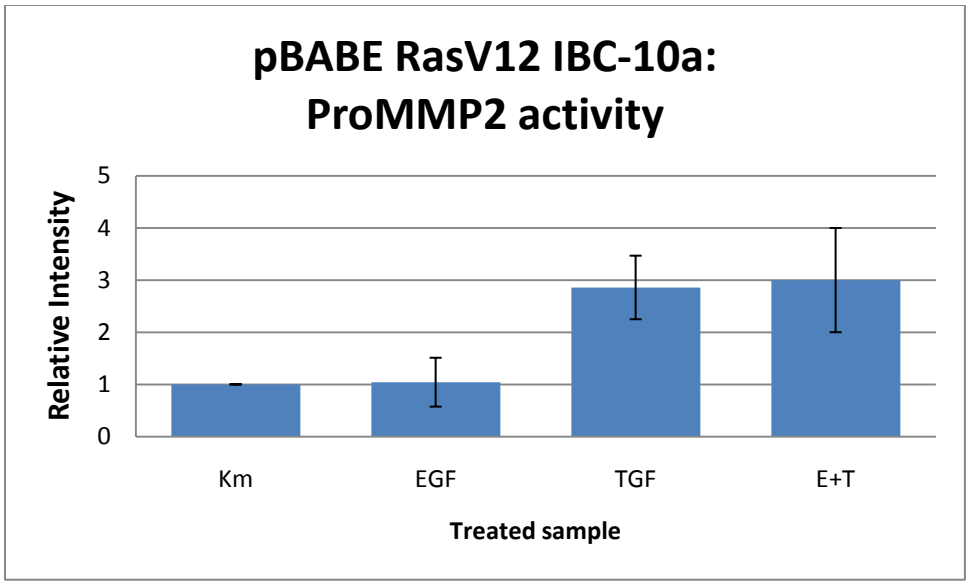


Figure 17: Graphs comparing the pro-MMP2/9 activities in untreated and treated (EGF, TGFβ1 and E+T) pBABE Ras V12 IBC-10a cells The above graphs compare the pro-MMP2/9 activities in untreated (Km) and treated (EGF, TGFβ1 and E+T) pBABE Ras V12 IBC-10a cells. Data represents the mean ± SD of 3 replicate experiments. The error bars in both graphs represent the standard deviation of the samples. The above activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was obtained by dividing their activity values by the Km activity value. a. The graph in this figure shows the increasing trend of RI of proMMP9 in pBABE RasV12 IBC-10a cells treated with TGFβ1 and E+T, where both exhibit very similar RI. Pro-MMP9 secretion in Km samples indicates the basal levels in the pBABE RasV12 IBC-10a cells. * indicates a significance ($p<0.0256$) in one way ANOVA with a Tukey post hoc (Groups are represented as A,B,B; A=EGF, B=TGF,E+T) b. The graph in this figure shows the increasing trend of RI of proMMP2 in pBABERasV12 IBC-10a cells treated with TGFβ1 and E+T, where both exhibit very similar RI. Pro-MMP2 activity in Km samples indicates the basal levels in the pBABE RasV12 IBC-10a cells.

3. Identification of the Ras pathway that cooperates with TGFβ1 to enhance MMP production and activity

The treatment of Ras cells with TGFβ1 when compared to E+T showed that both treatments yielded very similar proMMP9 and proMMP2 activities. These results suggest cooperation between one of the Ras pathways and TGFβ1 in order to drive Ras cells into an invasive phenotype as exhibited by parent cells treated with EGF and TGFβ1. The Ras mutants C40, G37 and S35 that activate the PI3K, RalGDS and the

MEK pathways respectively were treated with TGFβ1 for 7 days and collected after an overnight treatment of TGFβ1. Figure 18a compares the proMMP2 and proMMP9 activities in these Ras mutants treated with TGFβ1with respect to normal Ras cells.

Measurement of MMP2/9 secretion using Gelatin Zymography

Gelatin zymography revealed that the S35 Ras mutant induced maximum proMMP2 and proMMP9 activities when compared to the other Ras mutants. Interestingly the proMMP2 and proMMP9 activities in the G37 constructs were lower than their activities in normal Ras cells as well as other Ras mutant constructs. This trend is more obvious in the zymograms for proMMP9 bands than the proMMP2 bands. The proMMP2 activity is also much lower in all Ras mutants compared to the proMMP9 secretion, evident from the low signal intensity seen in Figure 18a.

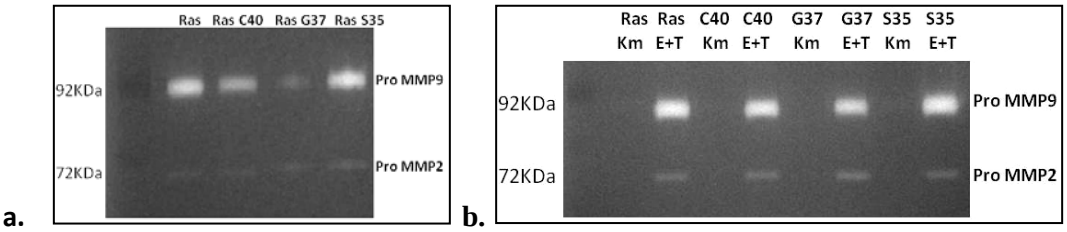
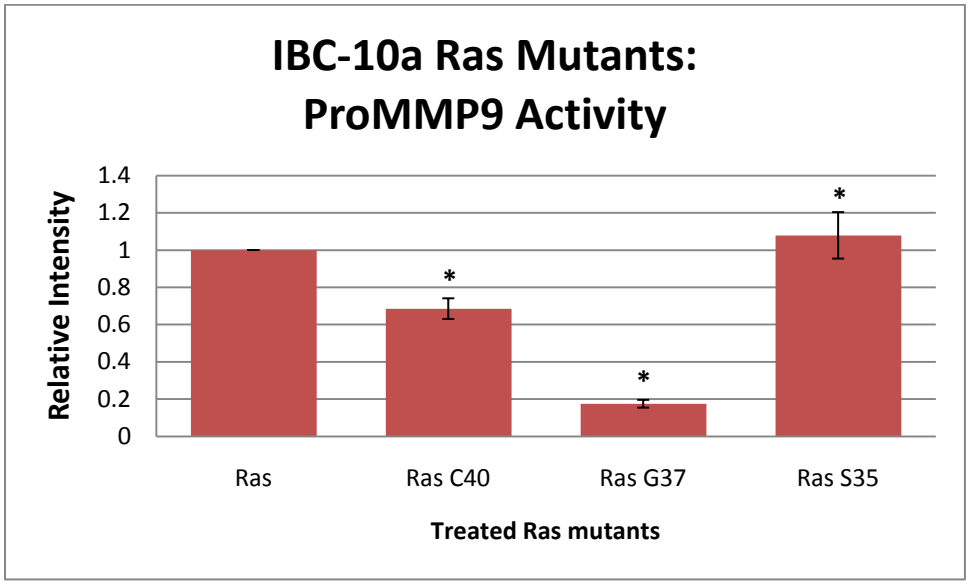


Figure 18: Gelatin Zymogram of IBC-10a Ras mutants treated with TGFβ1and E+T. Above image shows a zymogram of IBC-10a Ras mutants treated with TGFβ1(10ng/ml) and E+T (10ng/ml) at for 7 days. Each well was loaded with 0.01ug of the concentrated media collected at the end of the 7 day treatment. The media was changed every 3 days. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. a. The Ras mutants compared to normal Ras cells include C40 (PI3K activation), G37 (RalGDS activation) and S35 (MEK activation). The Ras S35 shows maximum gelatin digestion and thus maximum proMMP2/9 secretion compared to the rest of the samples, while the Ras G37 shows the least ProMMP9 secretion. b. Gelatin Zymogram of IBC-10a Ras mutants comparing E+T (5ng/ml) treatment to the negative control (Km) for 7 days. The E+T treatment boosts proMMP9 and proMMP2 activities in all four samples, while the Km samples exhibit no MMP2/9 secretion. All the Ras samples showed very similar proMMP9 and proMMP2 activities.

It was also important to analyze the basal MMP levels in the Ras mutants along with the effect of a combined EGF and TGFβ1 treatment on them. The zymogram in Figure 18b compares Km and E+T treatment in pBABE RasV12 IBC-10a, pBABE RasC40 IBC-10a, pBABE Ras G37 IBC-10a and pBABE RasS35 IBC-10a mutants. When grown in Km, all four cell types had no proMMP2 and proMMP9 activities whereas the E+T treatment boosted the activities of both proMMPs in all four Ras mutants.

All the Ras mutants were normalized against the pBABE RasV12 IBC-10a cells (negative control). It is evident from the graph (Figure 19a) that the Ras S35 mutant showed the highest proMMP9 secretion with a RI of 1.07, very similar to that of the control cells (RI of 1). The pBABE RasG37 IBC-10a mutants on the other hand showed the least amount of proMMP9 secretion with a RI of 0.17. These results recapitulate the band intensity analysis used in gelatin zymography.

a.



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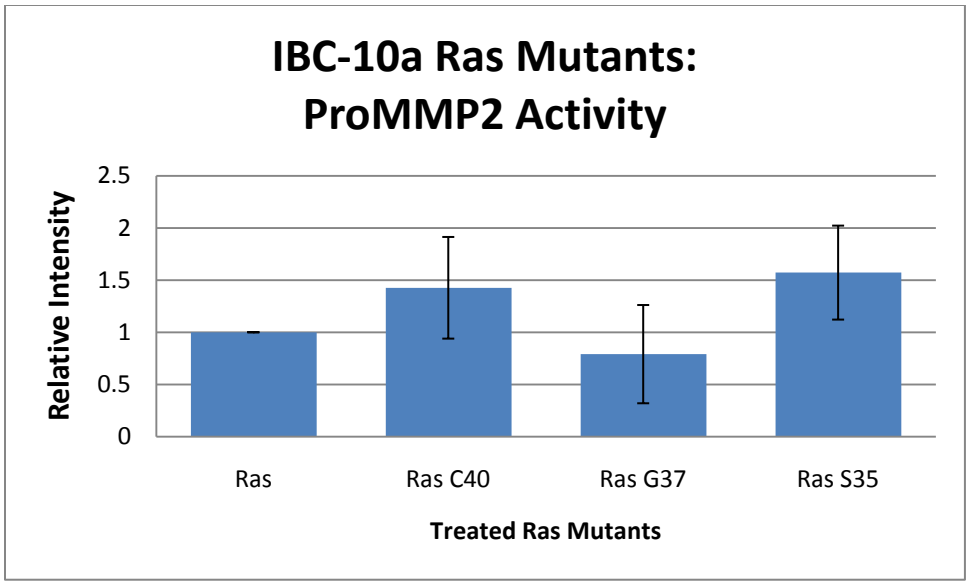
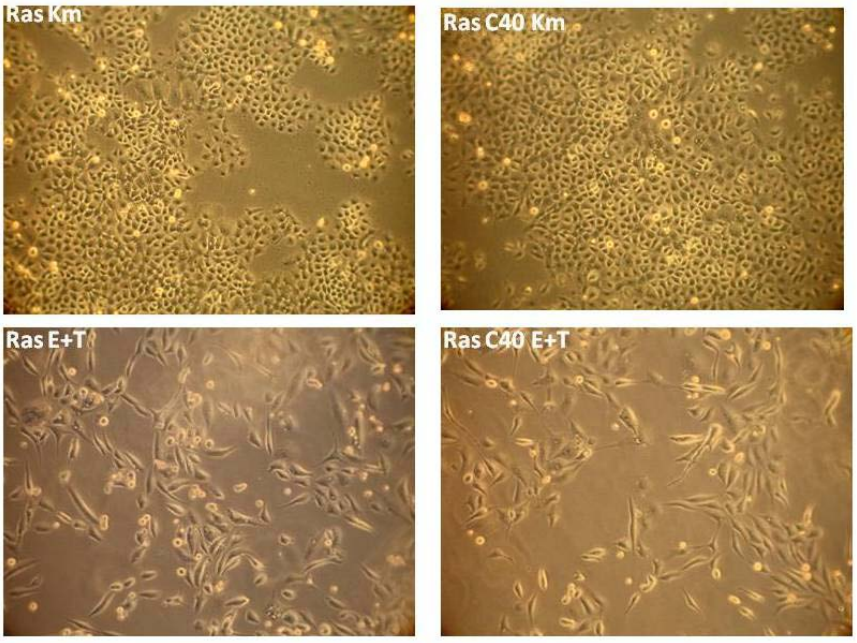


Figure 19: Graphs comparing the proMMP9 and proMMP2 activities in the Ras mutants The above graphs compare the proMMP9 and proMMP2 activities in the control pBABE RasV12 IBC-10a Ras cells and IBC10a Ras mutants- C40 (PI3K activation), G37 (RalGDS Activation) and S35 (MEK activation) treated with TGFβ1. Data represents the mean ± SD of 3 replicate experiments. The above activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was obtained by dividing their activity values by the control Ras activity value. a. The relative intensities of proMMP9 are compared among the Ras mutants. The RasS35 construct has the maximum proMMP9 secretion when compared to other Ras mutants and this value is equal to the proMMP9 secretion in the normal Ras cells (Control). Ras G37 construct shows the least proMMP9 secretion. * indicates significance ($p<0.0256$) in a one way ANOVA with a Tukey post hoc test. (Groups represented as A,B,C; A= RasC40, B=RasG37, C=RasS35) b. The graph compares the proMMP2 activities in normal IBC-10a Ras cells and 10aRas mutants, C40 (PI3K activation), G37 (RalGDS Activation) and S35 (MEK activation).There is a lack of significant difference in the proMMP2 RI of the Ras mutants. The trend of lower RI in Ras G37 construct compared to other mutants is maintained. Statistical analysis performed using one way ANOVA and a Tukey's post hoc test with a $p<0.0256$.

The quantified results of the proMMP2 activities in the Ras mutants did not show striking differences among the Ras mutants. Although the highest RI of 1.57 was observed in the pBABE RasS35 IBC-10a construct, the other mutants displayed similar proMMP2 activities (Figure 19b)

Appearance of an EMT phenotype

After the 7 day treatment with 10ng/ml of E+T, phase contrast images of the Ras mutants were obtained (Figure 20). These images help in observing the induction of EMT like phenotype in these mutants due to the E+T treatment. The cells in Km maintain the epithelial phenotype identified by the cobblestone appearance and the clear presence of cell junctions. The E+T treatment induced several EMT characteristics noticeable with phase contrast microscopy in the Ras mutants like loss of cell junctions and spindle shaped morphology. Similar to the zymogram results, the E+T treatment in all four mutants resulted in very similar morphological changes.



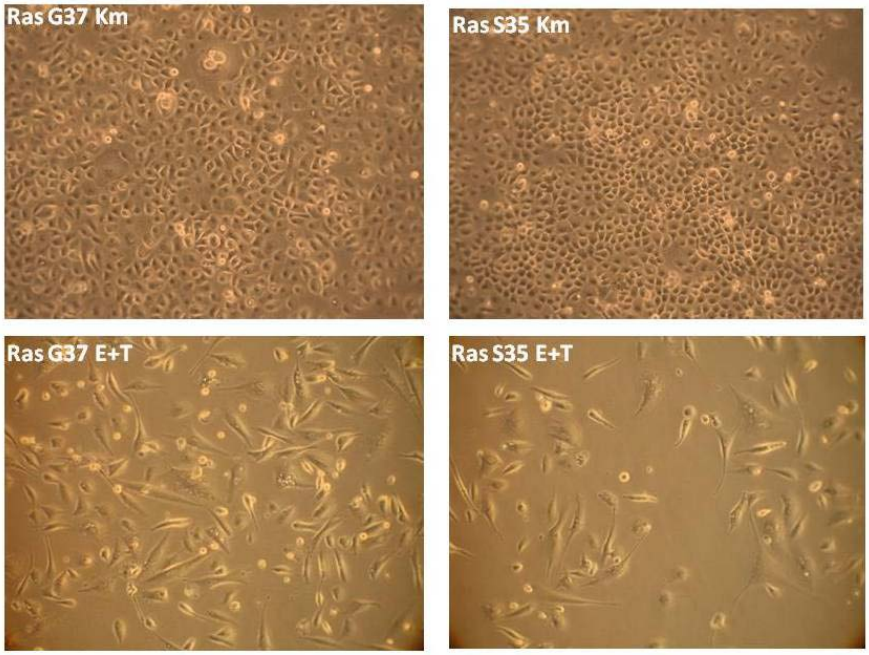


Figure 20: Comparison of the E+T treatment for in Ras mutants. The above image compares the E+T (10ng/ml) treatment for seven days in all four Ras mutants (pBABE RasV12 IBC-10a, pBABE RasC40 IBC-10a, pBABE RasG37 IBC-10a and pBABE RasS35 IBC-10a) with their respective Km controls. It is evident from these images that the E+T treatment induces an EMT phenotype, indicated by the fibroblast like appearance along with scattering and loss of cell junctions. The Km samples in each of the four mutants maintain their epithelial phenotype indicated by their cobblestoned morphology and the distinct cell junctions.

4. The inhibitory effects of EGCG on the E+T induced MMP2/9 secretion in primary prostate cancer cells and malignant prostate cells

Primary prostate cancer cells and malignant prostate cells were treated with EGCG to understand the effect of EGCG on the invasive capacity of cells based on their MMP2 and MMP9 production and activity. The media from these cells were collected and run on a zymogram to compare their MMP2 and MMP9 gelatinase activity. Accordingly, IBC-10a parent cells (pretreated with E+T for 9 days) were treated with

10uM, 30uM, 50uM of EGCG and untreated cells were used as the negative control. The untreated cells show maximum proMMP9 and proMMP2 gelatinase activities.

Measurement of MMP2/9 secretion using Gelatin Zymography

As seen in figure 21, the size and the signal intensity of proMMP9 and proMMP2 bands decrease with increasing EGCG concentrations. The untreated control sample exhibits maximum proMMP2/9 activities. Cells treated with 50uM EGCG show least proMMP2/9 gelatinase activities. Although 50uM of EGCG is required for a drastic decrease in proMMP9 secretion, the proMMP2 band disappears with 10uM EGCG treatment.

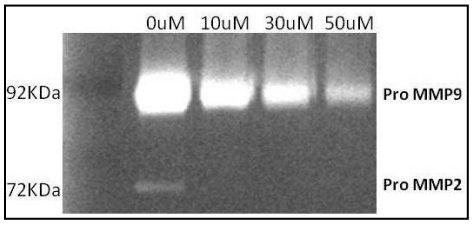


Figure 21: Gelatin Zymogram of IBC-10a cells treated with varying concentrations of EGCG. IBC-10a cells were treated with varying concentrations of EGCG (0, 10uM, 30uM, 50uM) overnight. Each well was loaded with 0.05ug of the concentrated media collected at the end of the overnight treatment. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. The untreated control sample exhibits maximum proMMP9/2 activities. The band intensity decreases with increasing EGCG concentrations. Cells treated with 50uM EGCG shows least proMMP2/9 activities. ProMMP2 band disappears with 10uM EGCG treatment.

The graph in figure 22 shows quantified data of proMMP9 activities in the IBC-10a parent cells treated with increasing concentrations of EGCG. The decreasing trend in the proMMP9 activities with increasing EGCG concentrations correlate with results in the zymogram images. As expected, the IBC-10a cells treated with 30uM EGCG has the

least relative intensity of 0.04 compared to the untreated sample (RI of 1) and 10uM treated sample (RI of 0.21).

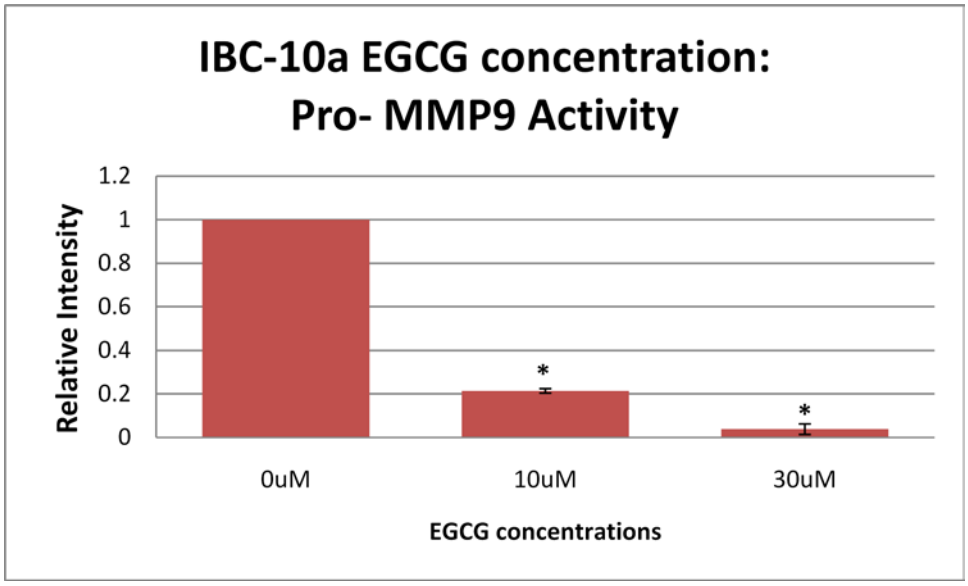


Figure 22: Graph comparing proMMP9 secretion in untreated and EGCG treated IBC-10a cells. The above graph compares the proMMP9 secretion in untreated and EGCG treated IBC-10a parent cells. Data represents the mean ± SD of 3 replicate experiments. The activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was calculated by dividing the activity value of each experimental sample by the untreated (negative control) activity value. The untreated sample shows the maximum proMMP9 secretion and the relative intensity values decrease with increasing EGCG concentration. 30uM EGCG shows the least proMMP9 secretion among the samples. A trend of decreasing proMMP9 activities with increasing EGCG concentration is observed. * indicates significance ($p<0.0256$) by one way ANOVA (Groups are significantly different and represented by A, B; A=10uM and B=30uM).

Next, PC3ML2 (malignant) cells were treated with different concentrations of EGCG (10uM, 30uM, 60uM and 100uM). Untreated cells were used as the negative control. Figure 23a shows a general decrease in proMMP2/9 activities with increasing EGCG concentrations suggesting a dose dependent MMP2/9 secretion in the malignant cells, a result observed in IBC-10a parent cells. Treatment with 10uM EGCG does not seem to affect the MMP2 and MMP9 secretions in PC3ML2 cells, but 30uM EGCG treatment

shows a reduction in proMMP2/9 activities as suggested by the decreased band signal intensity. 60uM EGCG showed the maximum reduction of the proMMP2/9 activities in malignant cells. Figure 23b shows a repetition of the EGCG treatment with 30uM and 60uM of EGCG which depicted similar trends of decreasing proMMP2 and proMMP9 activities.

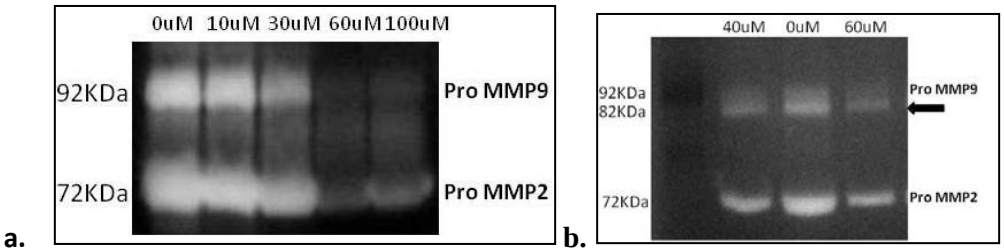
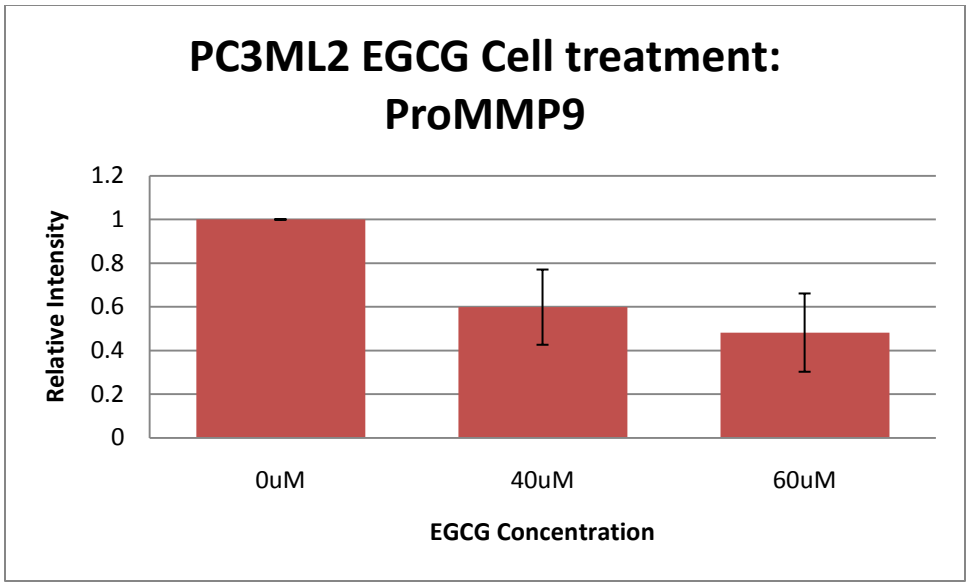


Figure 23: Gelatin Zymogram of PC3ML2 cells treated with varying concentrations of EGCG overnight. The size of the bands represents the level of gelatinase activity of the MMPs. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. a. The cells were treated with 10uM, 30uM, 60uM and 100uM of EGCG overnight. Each well was loaded with 0.1ug of the concentrated media collected at the end of the overnight treatment. The untreated control sample exhibits maximum proMMP9/2 activities. The band intensity decreases with increasing EGCG concentrations. Cells treated with 50uM EGCG shows least proMMP2/9 activities. b. The cells were treated with 40uM, 0uM and 60uM of EGCG overnight. Each well was loaded with 0.05ug of the concentrated media collected at the end of the overnight treatment. The untreated control sample exhibits maximum proMMP9/2 activities. The band intensity decreases with increasing EGCG concentrations. Cells treated with 60uM EGCG shows least proMMP2/9 activities. The arrow indicates the presence of an active MMP9 band.

The graphs in figure 24 display these results and they correlate with the trend observed in the zymograms. As expected, the PC3ML2 cells treated with 60uM EGCG has the least proMMP9 relative intensity of 0.48 compared to the untreated sample (RI of 1) and 30uM treated sample (RI of 0.6), seen in Figure 24a. Similarly, proMMP2 band intensity shows a gradual decline in activity with increasing EGCG concentrations (Figure 24b). The 60uM EGCG treatment resulted in least RI of 0.41 compared to the untreated sample (RI of 1) and the 40uM treated sample (RI of 0.55).

a.



b.

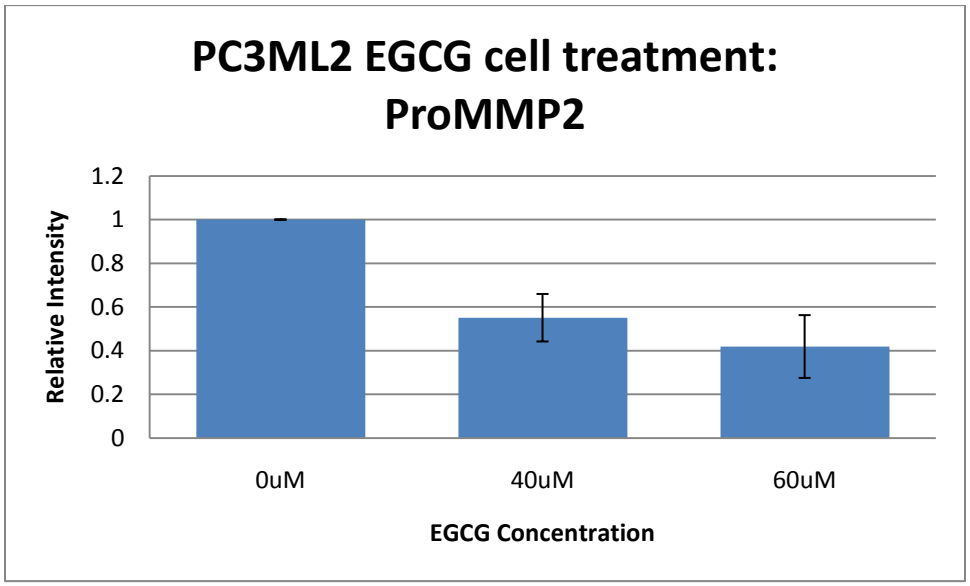


Figure 24: Graphs comparing the proMMP9 and proMMP2 activities in untreated and EGCG treated PC3ML2 cells. The above graphs compare the proMMP9 and proMMP2 activities in untreated and EGCG treated PC3ML2 (malignant) cells. Data represents the mean ± SD of 3 replicate experiments. The activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was calculated by dividing the activity value of each experimental sample by the untreated (negative control) activity value. a. The untreated sample shows the maximum proMMP9 secretion and the relative intensity values decrease with increasing EGCG concentration. 60uM EGCG shows the least proMMP9 secretion among the samples. b. The untreated sample shows the maximum proMMP2 activity and the relative intensity values decrease with increasing EGCG concentration. 60uM EGCG shows the least proMMP2 activity among the samples.

In order to understand whether EGCG was affecting MMP secretion or inhibiting enzymatic activity directly we collected the media from untreated PC3ML2 cells and IBC-10a cells and treated it with varying EGCG concentrations. The zymogram in Figure 25 shows decreasing proMMP2 activity with increasing EGCG concentration in the media obtained from IBC-10a cells. However, a significant difference in the proMMP9 secretion was not observed.

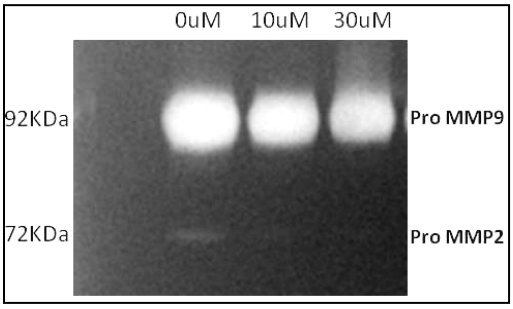


Figure 25: Gelatin Zymogram of the EGCG overnight treatment (0, 10uM, 30uM) of media obtained from untreated IBC-10a cells. The above image displays a zymogram of an EGCG overnight treatment (0, 10uM, 30uM) of media obtained from untreated IBC-10a cells. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. Each well was loaded with 0.01ug of the concentrated media collected at the end of the overnight treatment. The untreated control sample exhibits maximum proMMP2 activities, while the proMMP9 bands do not show significant difference in intensities. The proMMP2 band intensity decreases with increasing EGCG concentrations. Media treated with 30uM EGCG shows least proMMP2 activity.

The media treatment with EGCG was repeated on media obtained from untreated PC3ML2 cells. The media was treated with 0, 40uM and 60uM of EGCG overnight. Figure 26 displays the zymogram obtained from the above procedure. A slight decrease in the ProMMP2/9 activities was observed with increasing EGCG concentrations. The 60uM EGCG treatment rendered least ProMMP2/9 activities. However these results were not as significant as the effect of EGCG treatment directly on the cells (IBC-10a and PC3ML2).

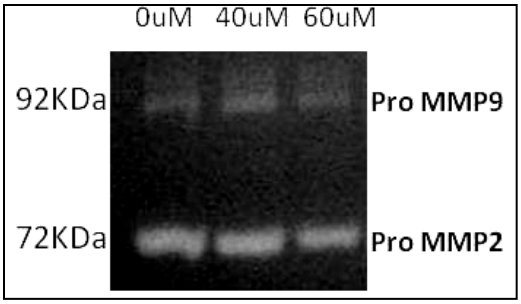
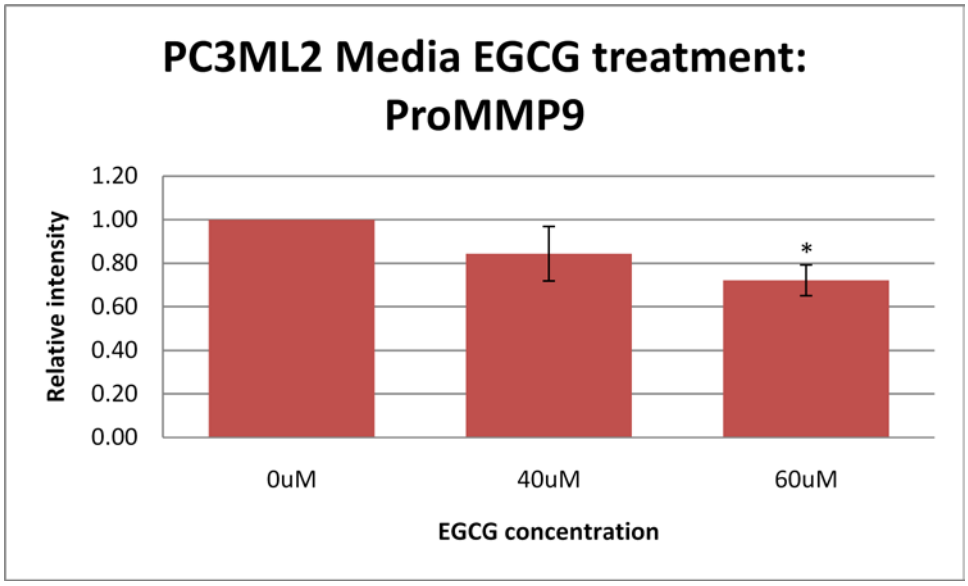


Figure 26: Gelatin Zymogram of the EGCG treatment of media obtained from untreated PC3ML2 cells. The above image displays a zymogram of the EGCG treatment (0uM, 40uM, 60uM) of media obtained from untreated PC3ML2 (malignant) cells. Each well was loaded with 0.05ug of the concentrated media collected at the end of the overnight treatment. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. The untreated control sample exhibits maximum proMMP2/9 activities. The proMMP2/9 band intensities decrease with increasing EGCG concentrations. Media treated with 60uM EGCG shows least ProMMP2/9 activities.

The graphs in Figure 27 show these quantified results and they display a decreasing trend in the proMMP9 and proMMP2 activities with increasing EGCG concentrations. Figure 27a shows that the 60uM EGCG treatment results in the least proMMP9 secretion with a relative intensity (RI) of 0.72 when compared to the untreated sample (RI of 1) and the 40uM EGCG treated sample (RI of 0.88). The quantified proMMP2 results are displayed in the graph in Figure 27b. Similar to proMMP9 results, the proMMP2 activity RI values show a decreasing trend with increasing EGCG concentration. The 40uM EGCG treatment had a higher RI value of 0.87 compared to the 60uM EGCG treated sample (RI of 0.62).

a.



b.

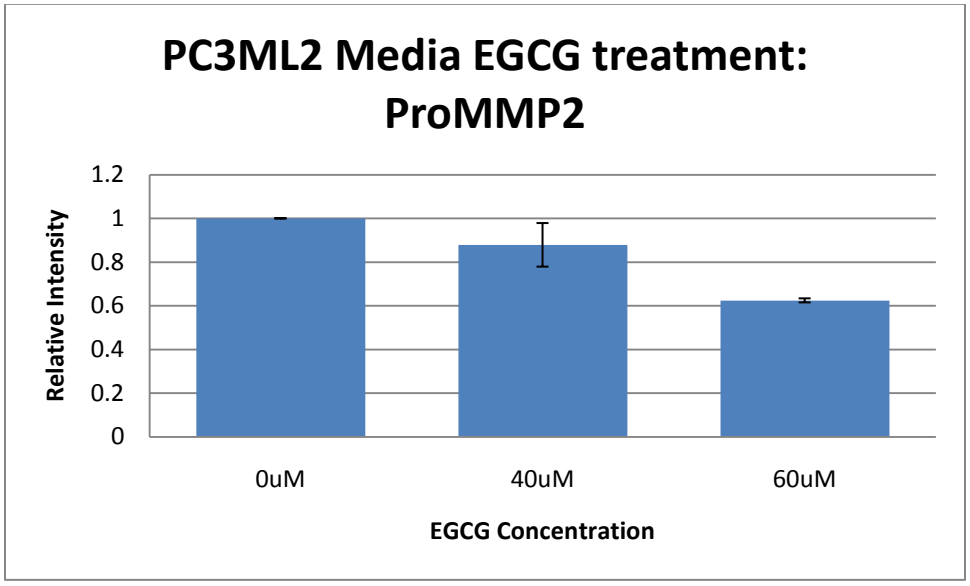


Figure 27: Graphs comparing the proMMP9 and proMMP2 activities in untreated and EGCG treated media from untreated PC3ML2 cells Data represents the mean + SD of 3 replicate experiments. The activity values were obtained by quantifying gelatin zymography data using Image J software. The relative intensity of the treated samples was calculated by dividing the activity value of each experimental sample by the untreated (negative control) activity value. a. The media from untreated cells was treated with 40uM and 60uM of EGCG. The untreated sample shows the maximum proMMP9 secretion and the relative intensity values decrease with increasing EGCG concentration. 60uM EGCG shows the least proMMP9 secretion among the samples.* indicates significance ($p<0.0256$) in one sample t-test (60uM treated group is different from the untreated group). b. ProMMP2 activities in media from PC3ML2 cells treated with increasing EGCG concentrations of 40uM and 60uM are represented here. The untreated sample shows highest proMMP2 activity and there is a decreasing trend in proMMP2 activities with increasing EGCG concentrations. 60uM EGCG shows the least proMMP2 activity among the samples.

The lack of significant differences in the proMMP2 and proMMP9 gelatinase activity of the media treated with increasing EGCG concentrations led us to analyze the effects of EGCG on the gels containing MMPs. The next set of experiments involved the incubation of acrylamide gels run with protein samples with 100uM of EGCG. The proteins obtained from media of PC3ML2 cells were run on acrylamide gels with gelatin. The gels were then incubated overnight in the absence and presence of 100uM EGCG. The Coomassie staining of these gels resulted in the zymograms in Figure 28.

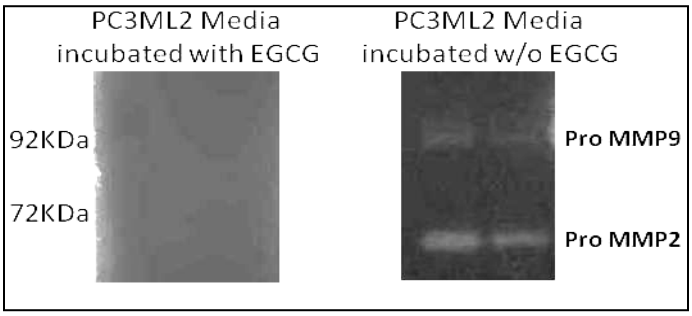


Figure 28: Gelatin zymogram obtained from incubating gels containing MMP2 and MMP9 with EGCG at 100uM concentration The zymogram on the right was incubated overnight in the presence of EGCG in the incubation buffer. There is no gelatinase activity observed in this gel. The figure on the left is the zymogram obtained by running the same proteins as run on the gel on the left (same concentrations) but in the absence of EGCG in the incubation buffer. In this figure, the proMMP9 and proMMP2 bands are clearly visible at 92kDa and 72kDa respectively.

Zymograms similar to the ones in figure 28 were obtained each time the experiment was repeated confirming the inhibition of gelatinase activity of proMMP2 and proMMP9 by EGCG. The lack of bands in the gel incubated with EGCG, made it difficult to quantify the results.

Chapter 5. Discussion and future work

Local invasion and metastasis are critical steps in cancer progression. One of the theories that elucidate the mechanism behind cancer metastasis is EMT- the transformation of epithelial cells to a mesenchymal phenotype. This process is

characterized by increase in mesenchymal markers like Vimentin, fibronectin and MMPs along with a downregulation of epithelial markers like E-cadherin, β catenin and laminin (Yang and Weinberg, 2008; Thiery, 2002; Polyak and Weinberg, 2009). The tumor microenvironment is a source of several factors that can induce EMT in primary tumors. Particularly, tumor associated stroma has been found to be a major source of several EMT signals originating from the mesenchyme (Polyak and Weinberg, 2009). Several growth factors like TGF, FGF and EGF reside in the stroma and are prospective inducers of EMT in cancers (Farrow et al, 2004; Hazelbag, 2002).

We have examined EMT induction in primary prostate cancer cell lines and for the first time have shown that a combination of EGF and TGFβ1 (E+T) can induce EMT via secretion of matrix metalloproteinases (MMPs). We have further shown that intact Ras signaling is essential for EMT to occur in primary prostate cell lines. We used Ras IBC-10a mutants with point mutations at C40, G37 and S35 which activated the PI3K, RalGDS and the MEK pathways respectively and inhibited the rest of the Ras pathways. The G37 construct inhibits the MEK and the PI3K pathways but activates the RalGDS pathway and was able to block MMP production. But a combined EGF and TGFβ1 (E+T) treatment of these mutant cells restored MMP production, clearly suggesting that an intact Ras pathway with MEK and PI3K activation was necessary for EMT induced MMP production in primary prostate cells. One of the primary goals of current cancer research is to identify agents that inhibit EMT, indirectly blocking invasion and metastasis. We have found that EGCG blocks E+T induced MMP secretion during EMT and may, therefore, be an invaluable therapeutic agent for blocking EMT events associated with invasion and metastases.

High levels of TGF β1 has been found in tumor stroma (Hazelbag et al, 2002) and inflammatory responses in cancer leads to the accumulation of EGF in the tissue surrounding the tumor (Farrow et al, 2004). Therefore, the combined action of these two ligands can induce an invasive phenotype in premalignant cancer cells. MMP2 and MMP9 play major roles in determining the invasive capacity of prostate cancer cells and are important prognostic markers of prostate cancer (Morgia et al, 2005). When studied individually, EGF and TGF β1 treatments have shown significant induction of MMP2 and MMP9. In ovarian cancer cells, EGF alone was found to induce MMP9 production in a PI3K dependent manner (Ellerbroek et al, 2001). The involvement of NF-KB downstream of the PI3K pathway has been supported by studies in breast cancer cells using NF-KB inhibitors and immunoflourescence results. The authors observed a considerable decrease in proMMP9 secretion with an NF-KB inhibitor and this suggests the presence of a NF-KB binding site on the MMP9 promoter region (Moulik et al, 2008; Le Page et al, 2005) It is well established that TGFβ1 alone can induce EMT in several forms of cancer. (Grunert et al, 2003). However, there are several pathways associated with TGFβ1 induced EMT. One of them seems to be its interaction with EGF and its downstream effectors like Ras.

It was necessary to identify standard concentrations of EGF and TGFβ1 for all the experiments in our studies. Optimal concentrations of EGF and TGFβ1 were identified using optimization experiments that used multiple combinations of the two factors on primary prostate cancer cells and analyzed their ability to secrete MMPs using gelatin zymography. Although, several studies in the past have conducted experiments with concentrations varying between 2ng/ml and 10ng/ml, our optimization studies revealed

very similar MMP2 and MMP9 gelatinase activities with 5ng/ml and 10ng/ml of EGF and TGFβ1 (Refer to Appendix A).

1. EGF and TGF induce EMT in primary prostate cancer cells:

The synergistic effects of growth factors, EGF and TGF β1, on the latent and active MMP2/9 activities of PCa-20a and IBC-10a parent cells are depicted in Figure 12 and Figure 13 respectively. The cells treated with Km served as the negative control and showed basal proMMP2/9 activities. It is clear from the prominent latent and active MMP2/9 activities in the E+T treatments that EGF and TGF β1 work together to enhance MMP2 and MMP9 production (Figures 12 and 13).

It is evident from figures 12 and 13 that the EGF treatment does not significantly induce proMMP2 and proMMP9 activities. On the contrary few studies have shown high MMP9 production and activity after EGF treatment of cancer cells (Kuo et al, 2009; Kim et al, 2009; Moulik et al, 2008). Although TGFβ1 alone was able to stimulate proMMP2/9 activities better than EGF alone, the combined treatment resulted in the highest gelatinase activities. Thus, our results indicate that EGF or TGFβ1 alone are not able to induce a prominent increase in proMMP2/9 activities and that a combined EGF and TGFβ1 treatment is necessary to observe this induction. The graphs comparing EGF, TGFβ1 and E+T treatments in primary prostate cancer cells indicate that TGFβ1 has more control on proMMP2 and proMMP9 production compared to EGF alone. This suggests that E+T as an additive effect on the MMP production in these cells, as the boost in the E+T treatment accounts for the sum of the effects of EGF and TGFβ1 on MMP production.

The lack of active MMP2 and MMP9 bands indicate the absence of MMP activators in the media obtained from treated primary prostate cancer cells. This suggests the failure of E+T treatment to induce MMP activators like MMP3, TIMP2 and MMP14 (Egeblad and Werb, 2002).

In addition to high MMP levels, the E+T treated cells also exhibited several EMT characteristics like spindle shaped morphology, loss of cell polarity, Vimentin expression and low E-cadherin expression (Refer to Appendix C). These results further indicate that E+T treatment induces EMT in primary prostate cancer cells.

The mechanism of the crosstalk between TGFβ1 and EGF is yet to be dissected. Based on the above findings and support from current literature we hypothesize the following mechanism of action of TGFβ1 with respect to its role in inducing MMPs (Figure 29). Binding of TGFβ1 onto its receptor activates the SMAD anchor for receptor activation (SARA) which functions to phosphorylate the SMAD2/3 complex. This step is followed by the addition of Cited 2 (CBP/p300- binding transcription co-activator) onto the SMAD2/3 complex and this complex enters the nucleus to activate the MMP9 promoter region. This proposed mechanism explains the upregulation of MMP9 with TGFβ1 treatment in the prostate cancer cells used in our study and has been suggested based on our experimental results along with data that supports the role of Cited2 in activating the MMP9 promoter (Chou et al, 2006).

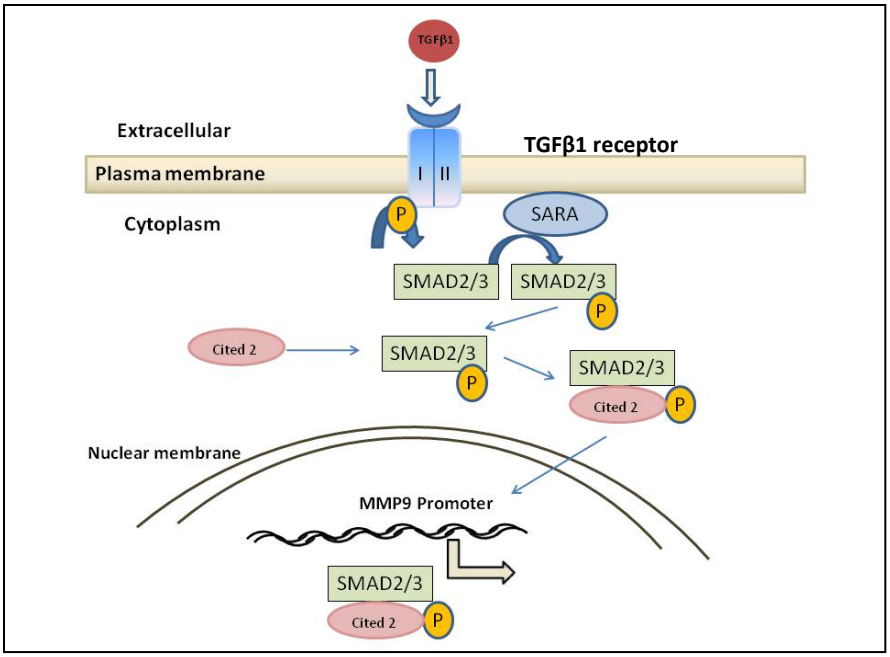


Figure 29: Proposed mechanism of action of TGFβ1 in the induction of MMP9 secretion. The activation of TGFβ receptor by TGFβ1 triggers the phosphorylation of SMAD2/3 by SARA. This activation step promotes the binding of CITED2 to the SMAD2/3 complex. This activated complex enters the nucleus to act upon the MMP9 promoter leading to MMP9 production and its further secretion. (Idea adapted from Chou et al, 2006)

Past studies have suggested possible mechanisms for MMP2 promoter activation via multiple transcription factors in cancer cells initiated by TGFβ1 treatment in cancer cells. Several results propose the activation of the MMP2 promoter by ATF2. The upregulation of MMP2 is accompanied by increased expression of p38 suggesting the activation of the p38/MAPK pathway after cancer cell treatment with TGFβ1. This pathway is Smad independent and suggests the role of ATF2 in the activation of MMP2 promoter. (Kim et al, 2007; Song et al, 2006; Kim et al, 2004) Figure 30 depicts the effect of TGFβ1 on the activation of p38 pathway and thus indirectly activating MMP2 promoter via ATF2 phosphorylation.

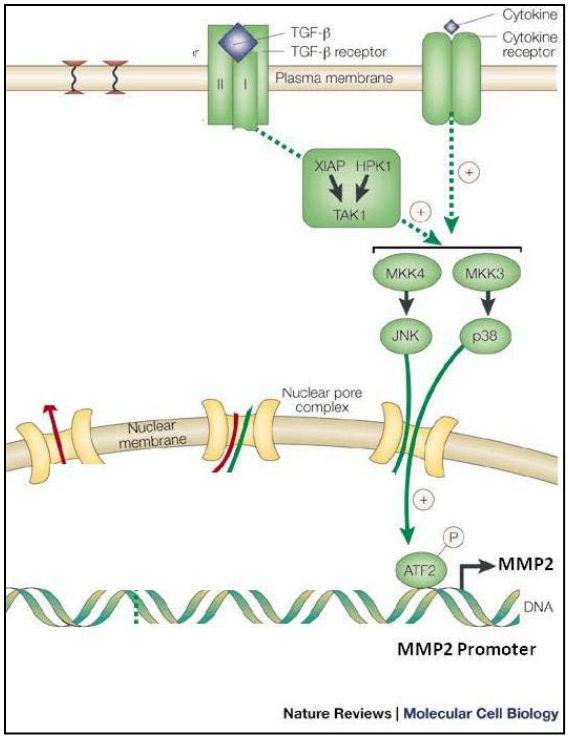


Figure 30: Proposed mechanism by which TGFβ1 activates the MMP2 promoter. Using a Smad independent mechanism, activation of the TGFR leads to the activation of the p38/MAPK pathway which in turn activates ATF2, a transcription factor found to directly bind to the MMP2 promoter region leading to its upregulation. (Adapted and modified from Massague, 2000)

2. *An intact Ras pathway for E+T induced EMT in primary prostate cancer cells:*

Although Ras mutations are not very common among prostate cancers, they are indirectly activated by several growth factor receptors that are often activated. Ras expression in prostate cancers is also associated with lowered androgen dependency for tumor growth, often associated with poor prognosis of prostate cancer. (Carey et al, 2007; Gioeli et al, 1999) The Ras Responsive element binding protein (RREB) was recently discovered to inhibit androgen receptor regulated transcription in prostate cancer cells. However, Ras overexpression in prostate cancer has the ability to inhibit this repressive

activity of RREB and results in increased PSA activity in prostate cancer cells (Mukhopadhyay et al, 2007). A recent finding shows that an oncogene- suppressor cascade, guided by a RasGAP gene (DAB2IP) promotes prostate tumor growth and metastasis. This gene functions as a “signaling scaffold” by regulating Ras and NF-KB. (Min et al, 2010)

It is well established that TGFβ1 is one of the inducers of EMT in cancer. As Ras is a downstream effector of EGF, the synergistic effect of EGF and TGFβ1 in increasing MMP production indicates a crosstalk between TGFβ1 and Ras signaling pathways.

Recently, Janda et al was able to show that Ras and TGF β cooperatively induce EMT in mammary epithelial cells. Ras transformation of the cells followed by TGF β treatment induced spindle shaped morphology, loss of epithelial markers and induction of mesenchymal markers. The transformation of these cells into the mesenchymal phenotype was permanent as identified by the lack of reversal of mesenchymal phenotype when TGF β1 treatment stopped. The study was able to show that only a Ras overexpression along with TGF β treatment can induce EMT and that TGF β treatment or Ras overexpression alone can only induce “cell scattering”. (Janda et al, 2002) Similar results were seen in Ras transformed hepatocytes with TGF β1 treatment. This study also observed growth inhibitory effects of TGF while inducing the conversion of hepatocytes to a fibroblastoid phenotype in the presence of activated Ha-Ras. (Gotzmann et al, 2001)

We observed that TGF β1 and E+T treatments induced very similar boosts in proMMP2 and proMMP9 activities in Ras transformed prostate cancer cells (Figure 16). The Ras mutation in these cells allows a constitutive expression of Ras and this eliminates the need of external activation of the Ras pathway by EGF, which is necessary

in parent cells that lack this mutation (as shown by E+T treatments in parent cells). Interestingly, the relative intensity of proMMP2 and proMMP9 of TGF β1 in Ras transformed cells were similar to the E+T treatments in parent IBC-10a cells. This observation suggests that the Ras mutation in the parent cells replaces exogenous EGF stimulation of the cells to induce proMMP2 and proMMP9 production.

Unexpectedly, higher protein loading in Ras cells gave rise to an active MMP2 band (Figure 16b) and this may indicate the presence of minimal MMP2 activators in Ras cells following TGFβ1 and E+T treatment. Current studies exogenously activate the proMMPs in the cells by treating them with APMA which cleaves proMMPs to release the active form (Ogata et al, 1992; Gum et al, 1997). However, we did not execute this step in our studies. We assumed that a general increase in proMMP gelatinase activity indicates a proportional rise in their active forms.

Treatment of Ras transformed cells with TGF β1 indicated a crosstalk between TGF β1 and Ras pathways. Further studies with Ras mutants helped dissect the specific Ras pathway that cooperated with TGF β1. Ras cells with mutation at C40, G37 and S35 were obtained which in turn specifically activated the PI3K, RalGDS and MEK pathways respectively, while inhibiting the other pathways in each case. Treatment of these mutants with TGF β1 showed that the S35 construct exhibited highest proMMP2/9 activities compared to the rest of the mutants, followed by the C40 mutant (Figure 17a). This result suggests that TGF β1 cooperates with the MEK and the PI3K pathways to induce EMT in the Ras transformed prostate cancer cells. Such a result has been seen in prostate cancer cells for the first time, although similar data has been shown in other cancers. More specifically, the co-operation between Ras/MAPK pathways and TGF β1/ Smad

pathways has been implicated in several cancers. Studies in breast cancer cell lines were able to show that TGF β1 treatment led to the loss of E-cadherin (marker of EMT) (Janda et al, 2002; Oft et al, 2002). Although a clear understanding of this interaction between the two pathways has not been achieved, experimental evidence indicate the need for both Smad signaling and MAPK for acquiring EMT (Yu et al, 2002; Itoh et al, 2003). A Ras mutant study similar to ours was conducted in fibroblasts. Implantation of these Ras mutants in mice revealed the critical role of the MEK pathway (RasV12S35) in generating metastatic subcutaneous and lung lesions accompanied by increase in ERK1/2 levels, a result not found in other Ras mutants they used (RasV12C40, RasV12G37) (Webb et al, 1998). Although majority of results in this area support MEK activated EMT induction and invasion (Bian et al, 2004; Yu et al, 2002), results seem to vary based on the cell type. A hepatocyte study that used an immortalized cell line showed the induction of a fibroblastoid like morphology in these Ras transformed cells with TGF β1 treatment, but also showed that MEK pathway was not involved in this process (Gotzmann et al, 2002).

Surprisingly, the G37 construct (RalGDS activation) in our study inhibited proMMP2/9 gelatinase activities signifying the role of the RalGDS pathway in inhibiting proMMP secretion. To date no work has been done on the RalGDS pathway that supports the above finding. RalGDS is a Ral guanine nucleotide exchange factor (RalGEF) that is responsible for the activation of GTPases like Ral. The effective metastasis in several cancers (lung, bladders and breast) can be seen upon activating the RalGEFs (like RalGDS) and studies show its dependence on the ERK pathway (Wolthius and Bos, 1999; Gildea et al, 2002). Contrary to this result, studies in breast cancer cell lines show

that the Ral pathway along with β arrestin can increase the migratory capacity by activating Lysophosphatidic acid (LPA) in breast cancer cells with higher tumorigenecity (Li et al, 2009; Li et al, 2008). The above results from the TGFβ1 treatment of the Ras mutants led us to investigate the effects of E+T on them. Figure 16b shows that a Km versus E+T treatment shows very similar proMMP2/9 activities among Ras mutants. Km alone fails to show any gelatinase activity in all four Ras mutants while E+T treatment induces proMMP2/9 activities in each cell type. Although the G37 construct seemed to inhibit MMP2/9 activation with TGFβ1 treatment, the E+T treatment did not deliver similar results. On the contrary it resulted in proMP2/9 activities very similar to the rest of the mutants, which implies that the RalGDS pathway does not interact with TGFβ1 to induce MMP production and that MEK and PI3K pathways are essential for TGFβ1 induced MMP production in Ras transformed prostate cancer cells. The G37 construct primarily activates the RalGDS pathway while inhibiting the other Ras pathways. The presence of EGF may have activated the endogenous Ras in these cells and this effect may have superseded the G37 mutation that blocked all Ras pathways except for the RalGDS pathway. Apart from directly indicating the pathways that cooperate with TGFβ1, the above Ras mutant studies elucidated that the induction of proMMP2 and proMMP9 by EGF alone could possibly occur via the MEK and the PI3K pathways.

3. EGCG inhibits MMP gelatinase activity:

Although the main focus of our study was to identify the effects of EGF and TGFβ1 on MMP2/9 secretion in prostate cancer cells, we were also interested in determining ways of counteracting local invasion and cancer metastasis via E+T induced MMP2/9 activation studies. One of the natural drugs in the forefront of herbal drug

cancer therapies is EGCG- a green tea extract that contains anti-tumorigenic flavanols. As an antioxidant, EGCG has been observed to play an important role in tumor repression and consequently has been proposed to be used in several cancer treatments. (Gupta et al, 2001; Suttie et al, 2003) The low toxicity of EGCG to normal, healthy cells and biased apoptotic effects on cancer cells makes it an attractive drug for chemotherapy. Due to multiple cellular targets of EGCG, it is difficult to identify a specific mechanism of action for this drug on cancer cells. The most commonly reported effects of EGCG in biological systems include its antioxidative effects, inhibition of activities related to tumor progression, reduction of cell growth, inhibition of cell adhesion to laminin and endothelial cells, etc. (Chung et al, 1999; Valcic et al, 1999; Isemura et al, 1993) Identifying the cellular pathways this drug particularly interacts with and inhibits is the main goal of many current studies. Dong et al was able to show the inhibition of activator protein- 1 (AP-1) via inhibition of the JNK pathway in mouse epidermal cells (Dong et al, 1997). EGCG was also recently found to prevent phosphorylation of EGFR, Stat3 and ERK proteins. As suggested by most EGCG studies, this study on head and neck cancer cells also concluded apoptotic activity in EGCG treated cells. (Masuda et al, 2001) Thus, multiple effects of EGCG on cancer cells have been recorded till date.

We conducted a dose response study of EGCG by treating premalignat cells (IBC-10a) and malignant (PC3ML2) cells with increasing EGCG concentrations. Figures 21 and 23 show a dose dependent decline in the proMMP2/9 gelatinase activities of cells treated with increasing concentrations of EGCG. A minimum concentration of 50uM of EGCG was required to dramatically decrease MMP2/9 secretion in PC3ML2 cells, whereas lower EGCG concentrations of 10uM and 30uM were enough to drastically

reduce proMMP2 and proMMP9 gelatinase activities in the IBC-10a cells. The IBC-10a (premalignant) cells were pretreated with E+T for 6 days before the EGCG treatment to induce an EMT phenotype in these cells. The PC3ML2 cells on the other hand were directly treated with EGCG and were not exposed to E+T treatments as they are highly malignant and express EMT characteristics under normal conditions.

Interestingly, premalignant cells were found to be more sensitive to EGCG induced proMMP2 and proMMP9 gelatinase activity. Altering vesicular transport in cancer cells is one of the proposed mechanisms for reduced MMP secretion with EGCG treatment. (Annabi et al, 2002) It can be hypothesized that malignant cells (PC3ML2) have developed better mechanisms to enable MMP transport to the plasma membrane along with its secretion. This could explain the need of higher EGCG concentrations to reduce proMMP2 and proMMP9 gelatinase activities in PC3ML2 cells, when compared to the premalignant (IBC-10a) cells.

One of the most comprehensive analyses of the effect of EGCG in invasion and gelatinase activity in prostate cancer has been done by Garbisa and colleagues. As seen in our study, they showed that an overnight cell treatment (neuroblastoma cells) with increasing concentrations of EGCG decreased the MMP2/9 activities in these cells. Their results indicated a drop in gelatinase activity at a concentration of 5uM although the Boyden chamber invasion studies rendered a similar drop in invasive activity at a lower range between 0.05uM and 0.15uM. It is surprising that this group obtained MMP gelatinase inhibition with EGCG at such low concentrations, even though their method of analysis is similar to our study.

The Garbisa group also conducted media treatments with EGCG and saw an inhibition of gelatinases with increasing EGCG concentrations, a result contrary to our data. In order to deduce the mechanism of action of EGCG, the group studied the effect of EGCG on gelatin and gelatinases and found EGCG to be preferentially binding to gelatinases rather than the substrate itself (gelatin). (Garbisa et al, 2001)

Apart from its role in inhibiting important pathways associated with EMT, EGCG has been found to inhibit MMPs by directly binding to them (Garbisa et al, 2001; Cheng et al, 2003). To analyze the effect of EGCG directly on the MMPs we treated the media from untreated PC3ML2 cells with increasing concentrations of EGCG at 37°C. Interestingly, the media collected from these untreated cells failed to show a drastic reduction in their gelatinase activities even with high EGCG concentrations (30uM) (Figures 25 and 26). This result was therefore contrary to the that obtained by Garbisa and colleagues, who were able to show preferential binding of EGCG to gelatinases at a low EGCG concentration of 1uM (Garbisa et al, 2001). It is questionable how this group saw such a drastic reduction in gelatinase activity of media treated with EGCG. The treatment of the PC3ML2 media with EGCG did not give us significant reduction in the gelatinase activity even at high EGCG concentrations of 60uM. Several studies have suggested the formation of EGCG- gelatinase complexes. (Cheng et al, 2003; Garbisa et al, 2001) However, the presence of SDS in the gels used for zymography may have affected the stability of these complexes. To circumvent this issue, we treated the gels containing MMPs with and without 100uM of EGCG overnight at 37°C. Contrary to the media treatment with EGCG, the gels incubated with EGCG were absolutely clear of any gelatinase activity compared to the gel incubated without EGCG, which showed both

proMMP2 and proMMP9 gelatinase activities (Figure 28). This result indicated the direct inhibition of proMMP2 and proMMP9 gelatinase activities by EGCG.

It is evident from the results obtained from our EGCG studies as well as those found literature that this “drug” uses several mechanisms to inhibit MMP gelatinase activity in cancer cells. The cell and gel (containing MMPs) treatments of EGCG suggest two possibilities- prevention of secretion of MMPs or direct inactivation of MMPs by forming EGCG-gelatinase complexes. Although studies have indicated the role of EGCG in transcriptional inactivation of MMPs, our supplementary results showed no change in MMP2 protein levels in cells treated with EGCG, eliminating this potential mechanism of EGCG in our studies (data not shown).

The debate on the mechanism of action of EGCG on MMPs continues as several studies show contradictory results. In 2004, Vayalil and Katiyar were able to show that EGCG inhibits MMP2 and MMP9 expressions in prostate cancer cell lines by repressing MAPK pathways which in turn affects the downstream target NF-KB, a factor claimed to be responsible for MMP production. The results from this study suggest that EGCG inhibits the transcription of the MMPs by deactivating the MAPK pathway, particularly by blocking the phosphorylation of ERK1/2 along with p38. (Vayalil and Katiyar, 2004) The hypothesis that EGCG prevents MMP secretion is supported by results obtained by Annabi and colleagues. Their results suggest that EGCG prevents MMP2 secretion in prostate cancer cells and it does so by inhibiting MT-MMP1 dependent MMP2 activation. (Annabi et al, 2002) We can also speculate that the inhibition of secretion could occur due to changes in the membrane organization and the formation of EGCG-gelatinase complexes.

We also observed several morphological changes in the cells treated with EGCG apart from its effects on MMP activation. However, after an overnight treatment, cells shrank and exhibited cell membranes with serrated edges. Several cells rounded up and were found floating in the media. EGCG induced at least 15% apoptosis with an overnight EGCG treatment of 30uM in IBC-10a cells, whereas EGCG treatment on human fibroblasts (0-50uM) showed lack of apoptosis (Refer to Appendix D). These results are similar to Paschka et al that reported apoptotic activity in prostate cancer cell lines treated with EGCG (Paschka et al, 1998).

It is evident from the above results that EGCG can inhibit MMP gelatinase activity and can thus be potentially used to block EMT. This can be determined by analyzing the expression of Vimentin, E-cadherin and other EMT markers using Western blotting along with RT-PCR data to observe changes at the mRNA level of the above proteins.

The stability of EGCG within experimental conditions and after ingestion has been well studied. Most studies have indicated the high sensitivity of this flavanol to a number of factors most relevant to our study like pH, partial pressure of oxygen and temperature. Sang et al have shown that auto-oxidation and epimerization are two main routes of EGCG degradation and instability. (Sang et al, 2005) Although our initial studies with EGCG gave drastic results clearly suggesting its role in inhibiting MMP2/9 activities, two months of storage lowered the efficacy of the drug in reducing MMP gelatinase activities in cell and media treatments (Figure 23a versus Figure 23b). Noting this reduction in its efficiency, we tested higher concentrations of the drug (40uM and 60uM compared to the 10uM and 30uM) on PCa-20a cells (pretreated with E+T).

Increased concentration of EGCG was able to provide results very similar to initial studies using lower concentrations (10uM and 30uM). This result indicated the degradation and loss of potency of the molecule over time. Low stability levels of this molecule may be responsible for this effect as is indicated by several other studies in literature. It was reported recently that considerable degradation of EGCG and other catechins present in tea occurs over a few months of storage, even in the absence of moisture and other obvious environmental factors like pH and temperature changes. This study that examined eight different commercial green tea leaves showed that EGCG is susceptible to degradation simply by storage within 2 months time. (Friedman et al, 2009) Thus, it is vital to use EGCG well within a few months in order to examine its highest efficacy in cancer treatment.

Chapter 6. Conclusion

In conclusion, the above results have indicated that firstly an intact Ras pathway is necessary for the E+T induced EMT in prostate cancer cells. Specifically, it is the activation of MEK and PI3K pathways that bolster the cooperation between the Ras pathway and TGFβ1. Additionally, the results from the EGCG studies have shown that this molecule has a high potential to be used as an inhibitor of MMPs. Although, the mechanisms of actions of this compound is still not clear, our results indicate its role in preventing proMMP2 and proMMP9 secretion from cells as well as direct inhibition of the gelatinolytic activities of proMMP2 and proMMP9. These results potentiate its role in combination drug therapies in prostate cancer.

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Appendix

Appendix A:

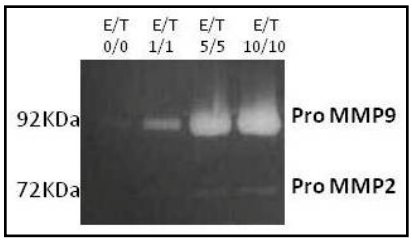
Optimization of EGF and TGFβ1 concentrations that induce EMT in PCa-20a cells.

PCa-20a parent cells were used to represent primary prostate cells for the optimization experiments. The cells were treated with various combinations of EGF and TGFβ1, as seen in the table below.

Table 6: Combinations of EGF and TGF β1 for optimization studies

	EGF	TGFβ1
1.	0ng/ml	0ng/ml
2.	1ng/ml	1ng/ml
3.	5ng/ml	5ng/ml
4.	10ng/ml	10ng/ml
5.	0.5ng/ml	10ng/ml
6.	10ng/ml	0.5ng/ml
7.	1ng/ml	10ng/ml
8.	10ng/ml	1ng/ml

The cells were treated with the combination of EGF and TGFβ1 for 9 days with a change of medium every 3 days. The media from the treated cells were collected on the 9^{th} day after an overnight treatment from the 8^{th} day. The media was concentrated using Amicon filters (10,000 MW limit). The protein concentration was analyzed of this collected media and run on a 10% acrylamide gel containing gelatin A (2ng/ml). The results were analyzed when gels were stained using Coomassie blue stain. The proMMP2/9 secretion bands of concentrations that showed maximum gelatinase activity were regarded as optimum concentration for the entire study.

a. 

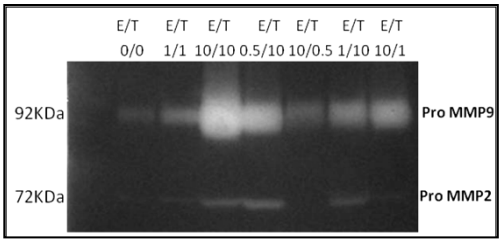
b. 

Figure 31: Comparison of pro MMP2/9 activities in 20a parent cells at different combinations of EGF and TGFβ1 concentrations. The upper bands in the zymogram show proMMP9 (92kDa) activity and lower bands show proMMP2 (72kDa) activity. The size of the bands represents the level of gelatinase activity of the MMPs. a. The proMMP2/9 activities of 5ng/ml and 10ng/ml of both EGF and TGFβ1 are the highest compared and are very similar to each other. b. The proMMP2/9 activities are highest in the 10ng/ml EGF and TGFβ1 treatment compared to all the other combinations of E+T concentrations. Each well of the gel was loaded with 0.05ug of protein in both figures.

The results from the E+T concentration comparison study in PCa-20a parent cells can be seen in figure 31. An important observation is the similarity in proMMP2/9 activities in cells treated with 5ng/ml and 10ng/ml of both EGF and TGFβ1. These concentrations also yielded the highest proMMP2/9 activities compared to the 0ng/ml and 1ng/ml concentrations of both ligands. Figure 31b shows these results and once again, 10ng/ml of both ligands produced maximum proMMP2/9 activities. The combinations of growth factors containing higher TGFβ1 concentration resulted in greater proMMP2/9 gelatinase activities suggesting the superseding importance of TGFβ1 over EGF in the induction of EMT via enhanced MMP activities. These results concur with the findings in the literature supporting the positive role of TGFβ1 in EMT induction in cancer cells (Lee et al, 2008).

The PCa-20a parent cells were treated with increasing concentrations of EGF and TGF β1 (1ng/ml, 5ng/ml and 10ng/ml) for seven days, with a change of

media every three days. Morphological changes in the treated cells were observed using phase contrast microscopy images. Increasing concentrations of E+T induce an EMT phenotype as seen in Figure 32. Various morphological changes like spindle shaped cells and lack of cell-cell junctions were evident with 5ng/ml and 10ng/ml concentrations of both EGF and TGF β1.

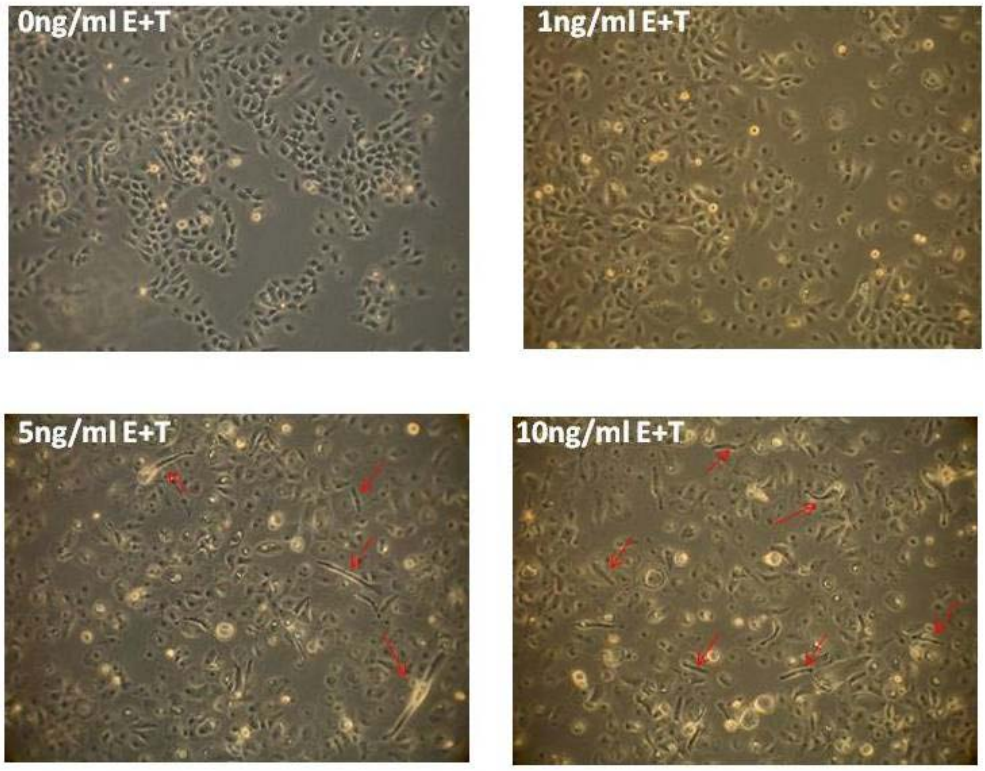


Figure 32: The gradual induction of an EMT phenotype in PCa-20a cells treated with increasing concentrations of E+T. The red arrows in the bottom images indicate the presence of elongated, spindle like cells that exhibit a fibroblast like appearance. Cells grown in Km were used as the negative control, where cells maintained cell junctions and have a cobblestone appearance of epithelial cells.

Appendix B

The effect of EGF and TGFβ1 on PCa-20a Ras cells

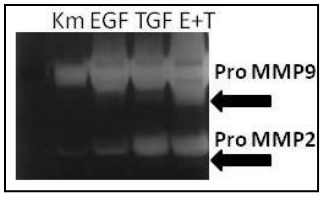


Figure 33: Gelatin Zymogram comparing proMMP2 and proMMP9 activities in PCa-20a Ras cells treated with Km, EGF, TGF and E+T for 9 days (all growth factors at 5ng/ml). It is evident from the proMMP2 activity results that both TGF and E+T treatments induce very similar MMP activities. The higher protein loading of 1.5ug enabled observation of the active MMP2 and MMP9 bands. These are indicated by black arrows.

Appendix C

Supplementary E+T induced EMT results

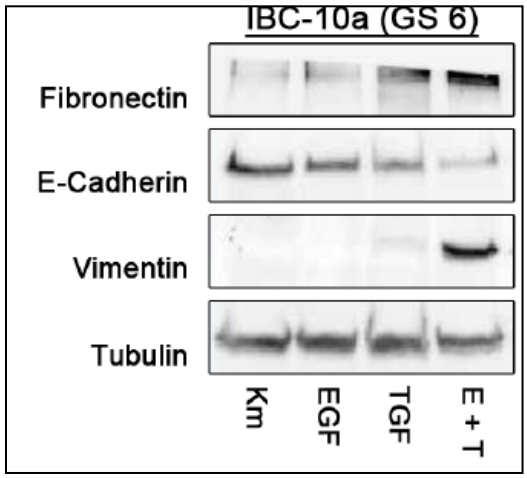


Figure 34: Western blot analysis of EMT markers in IBC-10a cells treated with Km, EGF, TGF and E+T. Tubulin was used as the loading control. Induction of mesenchymal markers like Vimentin and Fibronectin is observed with E+T treatment. There is also a simultaneous loss of E-cadherin indicative of lost epithelial features in the cells with E+T treatment. (Amatangelo, Personnel Communication)

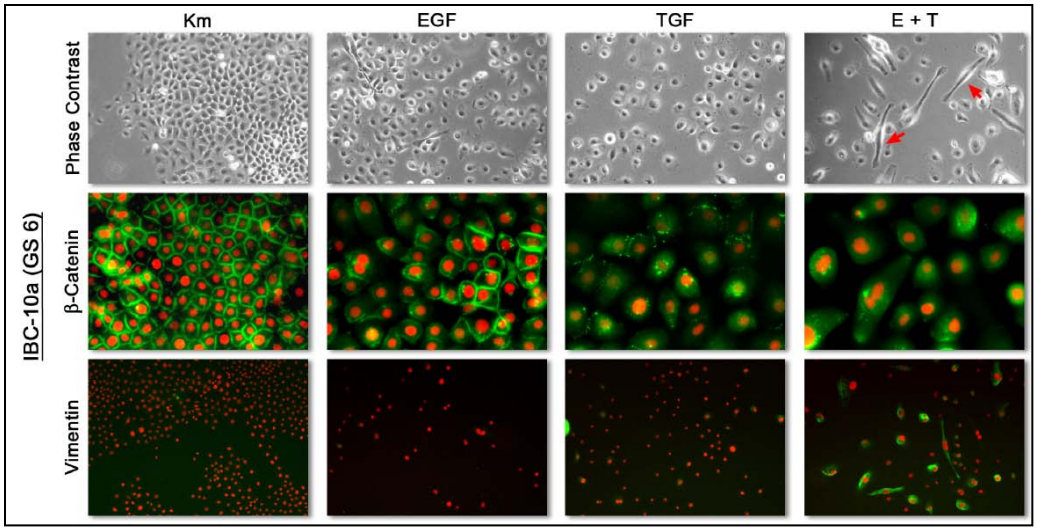


Figure 35: Phase contrast microscopy and immunostaining images of IBC-10a cells treated with Km, EGF, TGF and E+T (10ng/ml). The phase contrast images show the increase in scattering, loss of cell junctions and spindle shaped morphology of cells treated with E+T when compared to the Km for 7 days. The loss of β catenin and induction of Vimentin are indicative of conversion from epithelial to a mesenchymal phenotype in these cells with E+T treatment. Several EMT specific features are minimally seen in TGF alone treatments, but they get pronounced with E+T treatment. (Amatangelo, Personnel Communication)

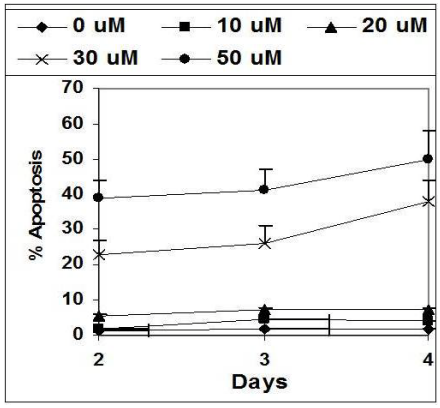
Appendix D

Apoptosis assays in healthy fibroblasts versus prostate cancer cells.

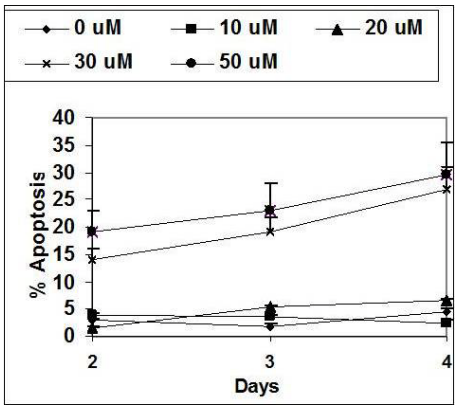
Apoptosis assays were conducted using Annexin V antibodies via flow cytometry. Results (Figure 36) showed that EGCG (0-50 uM) induced significant apoptosis in IBC-10a and PC3ML2 cells (Figures 36A and 36b). Concentrations of EGCG less than 20uM induced low levels of apoptosis of less than 5% in both cell types after 2, 3 and 4 days treatment. However, higher concentrations of EGCG (at 30uM and 50uM) induced significant apoptosis of more than 15% and 50% in both IBC-10a and PC3ML2 cells, respectively, by 2, 3 and 4 days (Figures. 36a and 36b). On the contrary, WI38 fibroblasts treated with similar EGCG concentrations showed that

both untreated and EGCG treated cells exhibited low levels of apoptosis (<2%) after 2, 3 and 4 days (Figure 36C).

a.



b.



c.

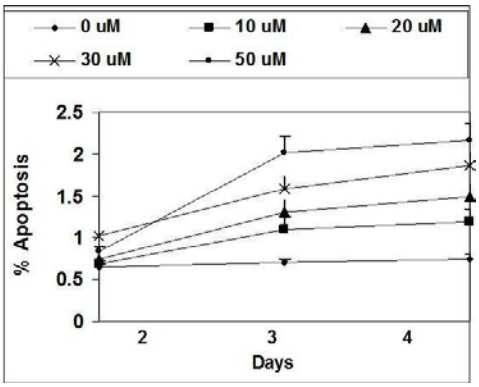


Figure 36: Comparison of percent apoptosis in IBC-10a, PC3ML2 and fibroblasts. Percent Apoptosis in IBC-10a cells. b. Percent apoptosis in PC3ML2 cells .c. Percent apoptosis in WI38 fibroblasts. All the above cells were treated with increased dosages of EGCG (0-50 uM) for 2, 3 and 4 days, respectively. Annexin V (Guava) labeling. (Stearns et al, in review)